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Biological mechanisms behind clozapine-induced agranulocytosis

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Academic Dissertation

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TIIVISTELMÄ

Klotsapiinin on osoitettu olevan tehokkain lääke hoidolle resistentissä skitsofreniassa. Klotsapiini saattaa olla myös muita psykoosilääkkeitä parempi, jos tehoa mitataan joillakin skitsofrenian hoidon osa-alueilla. Sen käyttöä rajoittaa kuitenkin vakava verenkuvaan liittyvä haittavaikutus, agranulosytoosi, jonka insidenssi on noin 0.8%. Klotsapiinin aiheuttaman agranulosytoosin tarkkaa molekyylitason syntymekanismia tai mekanismeja ei vieläkään tunneta.

Aiemmissa tutkimuksissa on osoitettu, että osalla skitsofreniapotilaista on piirteitä, jotka viittaavat autoimmuunihäiriöön. HLA- molekyylien (human leukocyte antigens) tiedetään assosioituvan lähes kaikkiin auto-immuunisairauksiin. Lisäksi klotsapiinin aiheuttaman agranulosytoosin on raportoitu assosioituvan useisiin HLA-molekyyleihin. Näiden löydösten perusteella halusimme tutkia kuinka HLA assosioituu klotsapiinin lääkevasteeseen ja klotsapiinin aiheuttamaan agranulosytoosiin. Potilaat jaettiin kolmeen eri ryhmään. Ensimmäisessä ryhmässä potilaat olivat saaneet hyvän vasteen ensimmäisen polven psykoosilääkkeestä (konventionaalisesta antipsykootista) (n=19). Toisen ryhmän potilaat eivät olleet saaneet vastetta ensimmäisen polven psykoosilääkkeestä mutta sen sijaan klotsapiinista (n=19). Kolmannen ryhmän potilaat olivat aiemmin saaneet klotsapiinihoidon yhteydessä granulosytopenian tai agranulo-sytoosin (n=26). Tutkimuksessa oli sekä sairaala- että avohoitopotilaita ja heille tehtiin skitsofreniadiagnoosi DSM-III-R kriteeristön mukaan. Suomalaiset terveet verenluovuttajat olivat kontrolleina (n=120). Havaitsimme, että HLA-A1 esiintyi merkitsevästi useammin potilailla, jotka eivät saaneet vastetta ensimmäisen polven antipsykootista mutta saivat vasteen klotsapiinista. Sen sijaan HLA-A1:n esiintymistiheys oli alhainen niillä potilailla, joille klotsapiini aiheutti neutropenian tai agranulosytoosin. Nämä tulokset viittaavat siihen, että HLA-A1 ennustaa hyvää hoitovastetta klotsapiinille sekä samalla osoittaa alhaista agranulosytoosin riskiä. Siksi HLA-tyypitystä voitaisiin käyttää avuksi valittaessa sopivia potilaita klotsapiinihoitoon. Tulokset voivat viitata myös siihen, että yhdessä skitsofrenian alaryhmässä HLA-A1 voi olla kytkentäepätasapainossa joidenkin altistavien geenien kanssa kromosomi 6:n MHC (major histocompatibility complex) -aluella. Nämä geenit voivat olla osallisena

säätelemässä psykoosilääkkeen vastetta ja klotsapiinin aiheuttamaa agranulosytoosia.

Tutkimme myös kuinka klotsapiini vaikuttaa geenien ilmentymiseen granulosyyteissä. Teimme mikrosiruanalyysin skitsofreniaa sairastavien potilaiden veren leukosyyteistä, kun he aloittivat ensimmäistä kertaa klotsapiinihoidon. Potilaat olivat hoidettavana sairaalassa ja heille tehtiin skitsofreniadiagnoosi DSM-IV-TR-kriteeristön mukaan (n=8). Potilaiden leukosyyttien geenien ilmentymisprofiileja verrattiin granulosyyttisten HL-60 (human promyelocytic leukemia) solujen geenien ilmentymismuutoksiin sen jälkeen, kun HL-60 soluja oli viljelty ilman klotsapiinia tai klotsapiinin kanssa. Tällä tavoin tunnistimme neljä geeniä, joiden ilmentymistaso oli muuttunut ja jotka liittyvät granulosyyttien kypsymiseen tai granulosyyttien apoptoosiin. Näitä geenejä olivat: MPO (myeloperoxidase precursor), MNDA (myeloid cell nuclear differentiation antigen), FLT3LG (Fms-related tyrosine kinase 3 ligand) ja ITGAL (antigen CD11A, lymphocyte function-associated antigen 1). Ilmentymismuutokset klotsapiinin aloittamisen jälkeen voivat viitata näiden neljän geenin osallisuuteen klotsapiinin aiheuttamassa agranulosytoosissa.

Koska on esitetty, että klotsapiini olisi sytotoksinen luuytimen stroomasoluille, tutkimme ovatko normaalit ihmisen luuytimen stroomasolut herkkiä klotsapiinille. Saimme viideltä vapaaehtoiselta luuytimen luovuttajalta luuydinnäytteet. Viljelimme normaaleja ihmisen luuytimen mesenkymaalisia stroomasoluja ja ihmisen ihofibroblasteja soluviljelmässä, jossa oli 10 µM muuntumatonta klotsapiinia tai hapettamalla bioaktivoitua klotsapiinia. Havaitsimme, että klotsapiini riippumatta bioaktivaatiosta oli sytotoksinen normaaleille luuytimen stroomasoluille primääriviljelmässä, kun taas samalla annoksella klotsapiini jopa stimuloi ihmisen fibroblastien kasvua. Löydös viittaa siihen, että suora luuytimen mesenkymaalisiin stroomasoluihin kohdistuva sytotoksisuus voi olla eräs mekanismeista, joilla klotsapiini aiheuttaa agranulosytoosin.

Uusia löydöksiä tutkimuksissamme oli se, että HLA-A1 voi määrittää skitsofrenian alaryhmän, jossa HLA-A1 voi olla kytkentäepätasapainossa psykoosilääkevasteeseen ja klotsapiinin aiheuttamaan agranulosytoosiin altistavien geenien kanssa. Klotsapiinihoidon aloittaminen muuttaa neljän spesifisen geenin ilmentymistasoa ja se voi viitata näiden geenien osallisuuteen klotsapiinin aiheuttamassa agranulosytoosissa. Osoitimme

myös, että klotsapiini on sytotoksinen ihmisen luuytimen mesenkymaalisten stroomasolujen primääriviljelmille ja siksi suora luuytimeen kohdistuva sytotoksisuus voi olla eräs mekanismeista, joilla klotsapiini aiheuttaa agranulosytoosin. Tulokset rohkaisevat lisätutkimuksiin, joissa voidaan tarkemmin selvittää klotsapiinin aiheuttaman agranulosytoosin mekanismeja ja löytää uusia mahdollisuuksia agranulosytoosin estämiseksi.

ABBREVIATIONS

ATP	adenosine 5’-triphosphate
BDNF	brain derived neurotrophic growth factor
BPRS	Brief Psychiatric Rating Scale
CD antigen	cluster of differentiation designation assigned to leukocyte cell surface molecules
cDNA	complementary DNA
CGI	Clinical Global Impressions
DMEM	Dulbecco's Modified Eagle's Medium (for cell culture growth)
DNA	deoxyribonucleic acid
DSM-III-R	Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision
EPS	extrapyramidal side effects
FasL	Fas ligand
FCS	Fetal calf serum (for cell culture growth)
FGA	first-generation antipsychotic
FLT3LG	Fms-related tyrosine kinase 3 ligand
GABA	γ-aminobutyric acid
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
GDB	the human genome database
HL-60 cells	human promyelocytic cells (that are used as a cell culture model for human leukocytes)
HLA	human leukocyte antigen
HSP	heat shock protein
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
IL-1Ra	interleukin-1 receptor antagonist
ITGAL	antigen CD11A, lymphocyte function-associated antigen 1
MHC	major histocompatibility complex
MNDA	myeloid cell nuclear differentiation antigen
MPO	myeloperoxidase; myeloperoxidase precursor
mRNA	messenger RNA
MSC	mesenchymal stromal cell
NADPH	nicotinamide adenine dinucleotide phosphate
NGF	nerve growth factors
NMDA	N-methyl-D-aspartate
NQO2	dihydronicotinamide riboside quinone oxidoreductase 2
PBS	phosphate-buffered saline
PCR	real-time reverse transcriptase-polymerase chain reaction
P-dATP	P-labeled deoxyadenosine 5’-triphosphate

RNA	ribonucleic acid
SD	standard deviation
SGA	second-generation antipsychotic
sIL-2R	soluble interleukin-2 receptor
sTNF-R	soluble tumor necrosis factor receptors
TD	tardive dyskinesia
TGF	transforming growth factor
TNF	tumor necrosis factor

1. ABSTRACT

Clozapine has proven to be the most effective therapeutic alternative in treating therapy-resistant schizophrenia and may even be superior to all other antipsychotics in several areas of schizophrenia treatment. However, its use is limited by a high incidence (approximately 0.8%) of a severe hematological side effect, agranulocytosis. The exact molecular mechanism(s) of clozapine-induced agranulocytosis is still unknown.

In previous studies a subgroup of schizophrenia has demonstrated aspects of an autoimmune process. Human leucocyte antigens (HLA) are known to associate with almost all autoimmune diseases. In addition, several HLA associations have been reported in clozapine-induced agranulocytosis. Based on these findings our aim was to investigate the mechanisms behind responsiveness to clozapine therapy and the associated risk of developing agranulocytosis by performing an HLA association study in patients who were grouped according to their responsiveness to therapy as follows: The first group comprised patients defined by responsiveness to first-generation (conventional) antipsychotics (n= 19). The second group was defined by a lack of response to first-generation antipsychotics but responsiveness to clozapine (n=19). The third group of patients had a history of clozapine-induced granulocytopenia or agranulocytosis (n=26). All patients were either hospital patients or outpatients meeting diagnostic criteria for schizophrenia according to DSM-III-R. Finnish healthy blood donors were used as controls (n= 120). We found a significantly increased frequency of HLA-A1 among patients who were refractory to first-generation antipsychotics but responsive to clozapine. We also found that the frequency of HLA-A1 was low in patients with clozapine-induced neutropenia or agranulocytosis. These results suggest that HLA-A1 may predict a good therapeutic outcome and a low risk of agranulocytosis and therefore HLA typing may aid in the selection of patients for clozapine therapy. Furthermore, in a subgroup of schizophrenia, HLA-A1 may be in linkage disequilibrium with some vulnerability genes in the MHC (major histocompatibility complex) region on chromosome 6. These genes could be involved in antipsychotic drug response and clozapine-induced agranulocytosis.

In addition, we investigated the effect of clozapine on gene expression in granulocytes by performing a microarray analysis on blood leucocytes of schizophrenic patients who had started clozapine therapy for the first time. These patients were all hospital patients meeting the diagnostic criteria for schizophrenia (DSM-IV-TR) (n= 8). The gene expression pattern of patient leukocytes was compared with gene expression alterations of granulocytic HL-60 (human promyelocytic leukemia) cells that were either treated or non-treated with clozapine. We were able to identify an altered expression in 4 genes implicated in the maturation or apoptosis of granulocytes: MPO (myeloperoxidase precursor), MNDA (myeloid cell nuclear differentiation antigen), FLT3LG (Fms-related tyrosine kinase 3 ligand) and ITGAL (antigen CD11A, lymphocyte function-associated antigen 1). The altered expression of these genes following clozapine administration may suggest their involvement in clozapine-induced agranulocytosis.

Since bone marrow stromal cells have been suggested as targets for clozapine-induced cytotoxicity, we investigated whether or not normal human bone marrow stromal cells are sensitive to clozapine. Bone marrow aspirates were obtained from five healthy volunteer donors. We treated cultures of normal human bone marrow mesenchymal stromal cells and human skin fibroblasts with 10 μM of unmodified clozapine and with clozapine bioactivated by oxidation. We found that, independent of bioactivation, clozapine was cytotoxic to normal bone marrow stromal cells in primary culture, whereas clozapine at the same concentration stimulated the growth of human fibroblasts. This suggests that direct cytotoxicity to bone marrow mesenchymal stromal cells is one possible mechanism by which clozapine induces agranulocytosis.

Our novel findings suggest that HLA-A1 may define a subgroup of schizophrenia where HLA-A1 may be in linkage disequilibrium with susceptibility genes involved in antipsychotic drug response and clozapine-induced agranulocytosis. We also found clozapine administration led to an altered expression in 4 specific genes that may be involved in clozapine-induced agranulocytosis. Finally, we showed that clozapine is cytotoxic to primary cultures of human bone marrow mesenchymal stromal cells, suggesting direct cytotoxicity to bone marrow as one possible mechanism by which clozapine induces agranulocytosis. Our findings provide the justification for further studies that could investigate the mechanisms of

clozapine-induced agranulocytosis more specifically and also focus on improved methods for its prevention.

2. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Lahdelma L, Ahokas A, Andersson LC, Huttunen M, Sarna S, Koskimies S. Association between HLA-A1 allele and schizophrenia gene(s) in patients refractory to conventional neuroleptics but responsive to clozapine medication. Tissue Antigens 1998, 51: 200-203.

II Lahdelma L, Ahokas A, Andersson LC, Suvisaari J, Hovatta I, Huttunen MO, Koskimies S. Mitchell B. Balter Award. HLA-A1 predicts a good therapeutic response to clozapine with a low risk of agranulocytosis in schizophrenic patients. Journal of Clinical Psychopharmacology 2001, 21:4-7.

III Lahdelma L, Jee KJ, Joffe G, Tchoukhine E, Oksanen J, Kaur S, Knuutila S, Andersson LC. Altered expression of Myeloperoxidase Precursor, Myeloid Cell Nuclear Differentiation Antigen, Fms-related Tyrosine Kinase 3 Ligand, and Antigen CD11A genes in leukocytes of clozapine-treated schizophrenic patients. Journal of Clinical Psychopharmacology 2006, 26:335-338.

IV Lahdelma L, Franssi S, Korhonen M, Andersson LC. Clozapine is cytotoxic to primary cultures of human bone marrow stromal cells. Submitted.

3. INTRODUCTION

Schizophrenia is a severe disabling mental illness of uncertain etiology affecting 0.5-0.9% of the population (Lewis et al. 2006b, Perälä et al. 2007). Schizophrenia is characterized by abnormal mental functions and disturbed behavior. These manifest characteristically in three classes of clinical features. Firstly, positive symptoms include delusions, hallucinations, and thought disorganization. Negative symptoms, as the second cluster, refer to the loss of motivation and emotional vibrancy. Finally, disturbances in basic cognitive functions are typically observed (Lewis and Lieberman 2000). The illness often starts with prodromal symptoms, and the onset may be insidious or rapid. Its course and outcome vary and the patients usually have remissions and exacerbations, but full recovery occurs only in a small minority (Kane 1996).

Schizophrenia is likely to be etiologically heterogeneous and probably a group of disorders (Coyle 2006). It also appears to be polygenic and is associated with environmental and developmental vulnerability factors (Lewis and Lieberman 2000). Although various psychosocial therapies are applied, so far, pharmacotherapy provides the foundation for treatment (Kane 1996, Mueser and McGurk 2004).

Current pharmacotherapy for schizophrenia includes two basic classes of medication, conventional (typical) or first-generation antipsychotics and atypical or second-generation antipsychotics. Although the pharmacological properties that confer the different therapeutic effects of the antipsychotic drugs have remained unclear, both classes of drug seem to act at least to some degree via the dopamine system(s), more specifically on dopamine D_2 receptors. Clozapine, an atypical drug, is an antipsychotic compound showing superiority over all current antipsychotic drugs. Despite decades of intense research, the mechanism underlying clozapine's distinctiveness in treating schizophrenia is not known. The superior qualities of clozapine are tempered by an approximately 0.8% incidence of agranulocytosis, a life-threatening condition (Alvir et al. 1993). The molecular mechanism(s) of clozapine-induced agranulocytosis has not yet been established (Dettling et al. 2007).

4. REVIEW OF THE LITERATURE

4.1. Neurobiological etiology of schizophrenia

The very first hypothesis of the pathogenesis of schizophrenia posited that it was related to disturbed serotonergic functioning in the brain. Only later, as the dopamine blocking activity of antipsychotics was discovered, was the dopamine hypothesis of schizophrenia formulated. It dominated biological schizophrenia research for decades postulating an overactivity of dopamine neurotransmission in the mesencephalic projections to the limbic striatum and suggested that the drugs achieved their antipsychotic efficacy by blocking the brain's dopamine D_2 receptors (Carlsson and Lindqvist 1963, van Praag et al. 1995, Haracz 1982). Recent studies have confirmed this, as the antipsychotic agents show a high affinity for striatal dopamine D_2 receptors, and the binding affinity correlates to their therapeutic efficacy (Miyamoto et al. 2005). The possible relation between serotonin and schizophrenia was revived by the special properties of clozapine and maintained until recently (Gonzalez-Maeso et al. 2008). The NMDA (N-methyl-D-aspartate) theory introduced the first nonmonoaminergic hypothesis of schizophrenia, implicating a dysfunction of the glutamatergic neurotransmission that is the major excitatory neurotransmitter in human brain (Olney and Farber 1995, Carlsson et al. 1997). The theory of glutamatergic dysfunction implicates the hypofunction of NMDA receptors. Blockade of NMDA receptors with drugs such as phencyclidine produces symptoms typically seen in schizophrenia, while agents that enhance NDMA receptor activity, such as glycine, selectively improve symptoms of schizophrenia (Goff and Coyle 2001, Kanahara et al. 2008). Moreover, there is evidence for abnormalities of the major inhibitory transmitter, GABA (γ-aminobutyric acid) (Lang et al. 2007, Reynolds and Harte 2007, Hashimoto et al. 2008). Some findings also implicate alterations in acetylcholine neurotransmission in schizophrenia (Sarter et al. 2005, Brooks et al. 2007).

Several studies have shown reciprocal synaptic relationships between the glutamatergic neuronal system and the forebrain dopaminergic projections (de Bartolomeis et al. 2005). In schizophrenia, an imbalance between dopaminergic and glutamatergic systems has been suggested in both cortical and subcortical areas. The functions of the neurotransmitter system are, however, complex and dysregulation by the illness or pharmacological

intervention in one system could alter neurotransmission in the other (Brooks et al. 2007, Laruelle et al. 2003). Therefore, the question remains whether alterations in neurotransmission are causative for the development of schizophrenia, or whether they are consequences of the disease or the treatment (Lang et al. 2007).

The current understanding of schizophrenia combines evidence from genetic, brain imaging, clinical, and pharmacological studies. Schizophrenia is most likely a heterogenous group of disorders sharing some phenotypic features, hence no single molecular event could completely explain the pathophysiology of the illness (Lewis and Lieberman 2000, Coyle 2006).

Vulnerability to schizophrenia has been related predominantly to genetic factors, since based on twin studies heritability (the percentage of variance explained by genetic factors) is estimated to be 80% and the concordance rate is around 70% (International Schizophrenia Consortium et al. 2009, van der Schot et al. 2009). Several putative susceptibility genes have been identified, including neuregulin 1 (NRG1), dystrobrevin binding protein 1 (DTNBP1), disrupted in Schizophrenia 1 (DISC1) and D-amino acid oxidase inhibitor (DAOA) (O'Donovan et al. 2009). The genetic mechanisms, however, still remain unknown. Novel studies focus on genes that are involved in pathways that can plausibly be related to hypotheses on the dysfunction of neurotransmission in schizophrenia (Sanders et al. 2008).

Schizophrenia is probably not a genetically defined static disorder but a dynamic process leading to dysregulation of multiple pathways (Lang et al. 2007). Genes are involved in the development and stabilization of cortical microcircuitry and could especially affect NMDA receptor-mediated glutamatergic transmission (Harrison and Weinberger 2005, Li et al. 2007). Epigenetic misregulation could also play a significant role, since a widespread DNA methylation defect has been suggested in the disorder (Huang and Akbarian 2007, Mill et al. 2008). Moreover, polymorphisms of some cytokine genes, such as IL-1B (interleukin), interleukin-1 receptor antagonist (IL-1-RA) and IL-10 have been associated with schizophrenia implicating immune deficits (Lang et al. 2007).

It has been proposed that severe, multiple and highly penetrant mutations may lead to schizophrenia. These mutations could be rare, and each of them individually responsible for schizophrenia in only one or a few

patients. Such mutations may dysregulate genes involved in neurodevelopmental pathways and contribute to the development of the illness (McClellan et al. 2007, Walsh et al. 2008). A recent genome-wide association study showed that common polygenic variation contributes to the risk of schizophrenia and implicated the major histocompatibility complex on chromosome 6p (International Schizophrenia Consortium et al. 2009). Furthermore, studies have shown altered mRNA levels in brain of patients with schizophrenia, suggestive of an involvement of several genes linked to the pathophysiology of cortical dysfunction (Akbarian and Huang 2006, Gibbons et al. 2009).

New avenues of research have been proposed in a recent hypothesis. It proposes that the evolutionary tug of war between the paternal and maternal genes could tip the brain development. A strong bias towards the paternal genes pushes a developing brain along the autistic spectrum and a bias towards the maternal genes along the psychotic spectrum increases the risk of developing schizophrenia later on, as well as mood disorders. The core of this hypothesis is that psychosis and autism represent two extremes on a cognitive spectrum with normality at its center. Social cognition is thus underdeveloped in autism, but hyper-developed to dysfunction in psychosis. The theory suggests that the development of these two diametric phenotypes is mediated in part by alterations in developmental and metabolic systems affected by genomic imprinting, notably via effects of genes that are imprinted in the brain and in the placenta (Crespi and Badcock 2008).

The hypothesis of schizophrenia involving deficiency of glial growth factors and synaptic destabilization suggests a functional deficiency of growth factors produced by glial cells including insulin, insulin-like growth factor I, neuregulin, tumor necrosis factor alpha; glutamate, NMDA, and cholinergic receptors (Moises et al. 2002). This hypothesis gets further support from several studies on brain white matter dysfunction or abnormalities in schizophrenia indicating a dysfunction of glial cells (Whitford et al. 2007, Chang et al. 2007, Karlsgodt et al. 2009).

There is strong evidence for the involvement of environmental factors in the pathogenesis of schizophrenia. These include exposure to infectious, autoimmune, toxic or traumatic insults, malnutrition and stress during gestation (Khashan et al. 2008, Sorensen et al. 2008, Insel et al. 2008,

Goldsmith and Rogers 2008). Also place of birth (urban environments) and birth in late winter have been associated with an elevated risk for schizophrenia (Susser et al. 1996, Mortensen et al. 1999). Patients with schizophrenia are also more likely to have a history of obstetrical complications (Geddes and Lawrie 1995, Isohanni et al. 2006).

Maternal environment may play a key role in schizophrenia. Maternal respiratory infections increase the risk of schizophrenia three- to sevenfold (Patterson 2007). There is also an association between elevated concentrations of cytokines or antibodies in maternal serum and incidence of schizophrenia in the offspring (Brown 2006). In rodents maternal influenza has been shown to cause abnormal behaviors in adult offspring mimicking those seen in schizophrenia, such as deficits in social interaction and working memory. Also the neuropathology in offspring is similar to that observed in schizophrenia (Patterson 2007).

Maternal infections during pregnancy – or direct fetal or early postnatal infection or hypoxia following obstetric complications - could influence brain development due to immune activation, possibly via circulating cytokines (Sorensen et al. 2008, Debnath and Chaudhuri 2006). Maternal infections induce pro-inflammatory cytokines which mediate the neurodevelopmental effects. In fact, the disruption of the fetal brain balance between pro-and anti-inflammatory cytokine signaling has been linked to disturbances in neural development (Meyer et al. 2009).

Extensive variations in the levels of inflammatory cytokines in the fetal environment may adversely affect the development of the nervous system and lead to disconnections. In animal models repeated hypoxia in brain regions involved in schizophrenia and prenatal immune activation during pregnancy have led to decreased NMDA receptor binding and maturation-dependent increased subcortical dopaminergic activity (Schmitt et al. 2007, Romero et al. 2008, Ozawa et al. 2006). In mice an association between prenatal immune activation and the emergence of behavioral dysfunctions in adulthood was critically dependent on the precise cytokine events taking place at the maternal–fetal interface (Meyer et al. 2008). Cytokines, including growth factors, neurotrophic factors, and cell differentiation factors may, as neurodevelopmental regulators, play a central role in brain development while regulating neuronal and glial migration, differentiation, and synaptic maturation (Nawa and Takei 2006). Increased levels of pro-

inflammatory cytokines have also been reported in the peripheral blood and cerebrospinal fluid of patients with schizophrenia (Sperner-Unterweger 2005).

As we later consider the possible associations between the HLA (human leukocyte antigen) system and schizophrenia, it is of interest to note that a non-classical HLA class I gene, i.e. HLA-G plays an important role during embryogenesis and may regulate the production of certain cytokines during early pregnancy. Maternal infection could lead to the disturbance of HLA-G expression and therefore, if HLA-G fails to maintain the immune homeostasis, the differentiation of the developing central nervous system could be affected (Debnath and Chaudhuri 2006).

Finally, the immunological hypothesis of schizophrenia indicates signs of inflammation in the central nervous system of schizophrenic patients. Here the evidence for immune dysfunction, however, is rather circumstantial than conclusive (Goldsmith and Rogers 2008). In patients with schizophrenia, findings suggest a non-specific activation of the inflammatory response (Sperner-Unterweger 2005) and a subgroup of patients may demonstrate signs of an autoimmune process (Strous and Shoenfeld 2006). Reports indicate that the balance of the immune response is shifted, type 1 (T_H 1) immune response is blunted whereas type 2 (T_H 2) immune response is increased (Lang et al. 2007, Goldsmith and Rogers 2008). Studies show that patients with schizophrenia have altered concentrations of both pro- and anti-inflammatory cytokines (Goldsmith and Rogers 2008), abnormal lymphocytes in peripheral blood and bone marrow (Hirata-Hibi and Fessel 1964), antibodies against nonspecific antigens, decreased levels of soluble intercellular adhesion molecules, and signs of increased permeability of the blood-brain barrier (Sperner-Unterweger 2005, Schwarz et al. 2000).

Genetic variation may increase the sensitivity to the teratogenic effects of prenatal infections or perinatal insults. As an example, studies in patients with schizophrenia show increased frequencies of specific polymorphisms (variants) of genes in the major histocompatibility complex known to influence the immune system, including *HLA-A10, -A11*, and *–A29* (Goldsmith and Rogers 2008). Furthermore, a genetic study showed an association between schizophrenia and the cytokine GM-CSF (granulocyte-macrophage colony stimulating factor) (*CSFRA*) and IL (interleukin)-3 receptor (*IL3RA*) abnormalities, suggesting that genetic variation in the

receptor structure or expression of proinflammatory cytokines may contribute to the risk of schizophrenia (Lencz et al. 2007).

Cytokine-mediated inflammatory response could be the common pathway by which varying environmental contributors such as infection, trauma, and anoxia might equally influence schizophrenia liability. The host's response would then be determined by genetic factors regulating the nature and degree of inflammation (Hanson and Gottesman 2006).

In conclusion, schizophrenia is a complex disorder. The disease is likely to be multifactorial and individual patients suffering from schizophrenia may present different biological subtypes. The greatest known risk for developing schizophrenia is a genetic susceptibility evolving from the addition or potentiation of a cluster of genes or multiple mutations with high penetrance (Walsh et al. 2008). Genetic vulnerability does not, however, necessarily lead to the disease. The current neurodevelopmental hypothesis of schizophrenia integrates causative genes and environmental influences. Altered neural development due to adverse events during fetal development or the early postnatal period may lead to dysregulation of multiple pathways contributing to disease manifestation during adolescence in the context of developmental maturation as a set of brain dysfunctions (Lewis and Lieberman 2000). However, the pathological cascade of schizophrenia is still not understood (Lang et al. 2007). Alterations in key neurotransmitter systems suggest that schizophrenia is characterized by overstimulation of subcortical dopamine D_2 receptors, hypoactivity of frontal cortical dopamine D_1 receptors, and reduced prefrontal glutamatergic activity (Lang et al. 2007). The alterations may originate from an early neurodevelopmental disturbance influenced by either genes or factors linked to placental environment.

4.2 Drug treatment of schizophrenia

4.2.1 First-generation antipsychotics

Treatment with antipsychotic drugs was invented by coincidence at the beginning of the 1950s. Chlorpromazine, the first antipsychotic drug, was initially used as an antihistamine as adjuvant to anesthetics during surgery. It soon spread into psychiatry, as it was reported to be efficient in treating acute psychosis (Delay et al. 1952). Subsequent studies confirmed its clinical efficacy. Since then, antipsychotic drugs have revolutionized the treatment of schizophrenia and other psychotic disorders. A number of other phenothiazine compounds including perphenazine were soon introduced, followed by various other agents affecting dopaminergic neurotransmission such as haloperidol. The development of the first-generation antipsychotics (FGAs) was based on the hypothesis that schizophrenia reflected a brain hyperdopaminergic activity and the drugs achieved their antipsychotic efficacy by blocking the brain dopamine D_2 receptors (Carlsson and Lindqvist 1963, Haracz 1982). Several decades after their introduction, the typical or conventional antipsychotics are still considered effective in treating the symptoms of schizophrenia (Jones et al. 2006, Lieberman et al. 2005, Leucht et al. 2008) and reducing the risk of relapse (Gaebel et al. 2007).

Several limitations in the use of the first-generation antipsychotic drugs prompted a search for newer agents. Around 20-25% of patients with schizophrenia fail to show a satisfactory response to conventional drug therapy, manifested as treatment resistance (Lewis et al. 2006a). In addition, these agents may not or only modestly improve the negative symptoms of schizophrenia (Meltzer 1999). Conventional drugs are also associated with a wide range of unwanted effects adversely influencing treatment adherence (Morrens et al. 2008, Kane 2006). These include acute neurological side effects (e.g. extrapyramidal side effects, EPS) or adverse effects following long-term exposure (e.g. tardive dyskinesia, TD).

4.2.2 Second-generation drugs

The synthesis of a novel dibenzodiazepine clozapine in 1958 heralded the introduction of a new class of drugs, the second-generation antipsychotics (SGAs), also referred to as atypical agents. Preclinical and clinical testing showed that clozapine has properties different from those of classic antipsychotics- most importantly, a relative lack of extrapyramidal symptoms (EPS) as well as a substantial therapeutic advantage (Alvir et al. 1993). However, reports on the severe side effect of agranulocytosis promptly restricted its widespread use (Idanpään-Heikkilä et al. 1975, Idanpään-Heikkilä et al. 1977).

Clozapine stimulated the development of new agents with comparable therapeutic and pharmacological profiles but with more tolerable side effects, such as risperidone, olanzapine, quetiapine, ziprasidone, and aripiprazole (Nasrallah 2007). These drugs rarely cause agranulocytosis but, like clozapine, have a lower risk of extrapyramidal side effects and tardive dyskinesia and were therefore described as atypical drugs (Melzer 1995). Today, they are considered the first-line treatment in schizophrenia. In addition, they may be more efficient in treating negative symptoms and cognitive disturbance in schizophrenia and show mood-stabilizing and mood-elevating effects (Tandon et al. 2008, Tandon and Fleischhacker 2005). SGAs may ensure better adherence and tolerability and therefore be better in preventing relapses (Kane 2008). No consistent differences in efficacy have been found between the second-generation drugs, other than a superior efficacy of clozapine in treatment-refractory schizophrenia (Kane et al. 1988, Tandon et al. 2008). Nevertheless, some novel studies have argued the superior efficacy of SGAs over FGAs. SGAs have, however, consistently showed lower risk of extrapyramidal side-effects (Lewis et al. 2006b, Tandon et al. 2008, Lieberman et al. 2005).

The pharmacological mechanisms underlying the various therapeutic properties of most atypical agents are not known (Miyamoto et al. 2005). Most atypical agents act via blockade of D_2 dopamine receptors but bind to numerous other receptors as well (Tauscher et al. 2004). The serotonin–dopamine (5-HT_2/D_2) antagonism theory postulates that a greater potency at the serotonin 5-HT_{2A} receptor relative to affinity to the dopamine D_2 receptor can predict atypicality and may explain the enhanced efficacy and reduced EPS liability (Meltzer et al. 1989). Most, but not all atypical agents

share this profile, indicating that whilst a combined dopamine-serotonin profile may provide atypicality it is not sufficient to explain it. As atypical agents are a heterogeneous group of drugs with distinct receptor profiles, the term "atypical" should be replaced by "second-generation antipsychotics" (Remington 2003, Fleischhacker and Widschwendter 2006).

The concept of regional selectivity assumes that blockade of dopamine D_2-like receptors (D_2, D_3 and D_4 receptors) in the limbic areas and temporal cortex reduces positive symptoms with a minimal blockade of striatal dopamine D_2 receptors, thereby minimizing the incidence of EPS. These mechanisms are consistent with the proposed anatomically selective effect of atypical antipsychotics (Kessler et al. 2005, Grunder et al. 2006, Hertel 2006). Also dopamine D_2 receptor partial agonists, such as aripiprazole, have been shown to improve both positive and negative symptoms of schizophrenia (Brennan et al. 2009).

So far, all effective antipsychotic drugs seem to occupy dopamine D_2 receptors to some degree. However, it is not known why some individuals with schizophrenia respond well to antipsychotic drug treatment while some are therapy-resistant, or why negative and cognitive symptoms seem to respond less well to antipsychotics than positive symptoms of schizophrenia (Laruelle et al. 2003).

4.2.3 Clozapine and its possible mechanisms of action

Clozapine is the most effective antipsychotic compound in treating therapy-resistant schizophrenia (Lewis et al. 2006a, Nasrallah 2007, Kane et al. 1988, Tauscher et al. 2004, Kane et al. 2001, Chakos et al. 2001). In addition, clozapine can improve cognitive deficits (Lewis et al. 2006b, Kane et al. 2001, Peuskens et al. 2005, McGurk 1999), has shown superior efficacy over other antipsychotics for positive symptoms (Carpenter and Buchanan 2008), causes a three-fold reduction in the risk of suicidal behavior in schizophrenic patients (Hennen and Baldessarini 2005) and may be associated with a lower mortality than any other antipsychotics (Tiihonen et al. 2009).

Chemically clozapine (piperazinyl-debenzo-[1-4]-diazepine) is related to the newer SGAs olanzapine and quetiapine and displays a broad spectrum of receptor affinity (Markowitz et al. 1999). Beyond this, clozapine has unique

effects on a variety of central nervous system receptors (Horacek et al. 2006). In animal models it works selectively on the mesolimbic dopaminergic system and is less active in the striatal dopaminergic neurons, which could explain its very low propensity for EPS and the low incidence/lack of occurence of TD (Elsworth et al. 2008). Clozapine´s diminished tendency to induce extrapyramidal symptoms has been attributed to a comparatively high serotonin 5-HT_{2A} receptor to dopamine D_2 receptor antagonism and its fast dissociation from the D_2-receptor (Tauscher et al. 2004, Kapur and Seeman 2001). In addition, clozapine has an affinity to several other receptors, including dopaminergic D_1, D_3 , D_4 , D_5 receptors, serotonergic 5-HT_{1A}, 5-HT_{1C}, 5-HT_{2A}, 5-HT_{2C} ,5-HT_3 , 5-HT_6, 5-HT_7 receptors, adrenergic α_1-, and α_2- receptors, histaminergic H_1, H_3, H_4 receptors and muscarinic M_1 and M_5 receptors (Markowitz et al. 1999, Horacek et al. 2006, Ashby and Wang 1996, Kinon and Lieberman 1996, Liu et al. 2001, Gunes et al. 2009).

The adverse effects of clozapine reflect its pharmacological properties. Orthostatic hypotension and sexual dysfunction are linked to adrenergic α-blockade, H_1-blockade may lead to sedation, and muscarinic M1-antagonism may cause anticholinergic effects such as constipation and tachycardia, blurred vision, and urinary retention (Markowitz et al. 1999). Moreover, metabolic abnormalities are linked to 5-HT_{2A} and 5-HT_{2C} receptor blockade (Gunes et al. 2009). Other common adverse effects include dizziness, transient eosinophilia, hypersalivation, hyperthermia, leukocytosis, nausea and seizures (Wagstaff and Bryson 1995). Clozapine-induced suppression of granulocyte series can result in leukopenia, neutropenia or, most severely, agranulocytosis (Pirmohamed and Park 1997). The wider use of clozapine is restricted due to its propensity to cause agranulocytosis in about 0.8% of patients. Other rare but serious adverse reactions associated with clozapine include neuroleptic malignant syndrome, myocarditis, cardiomyopathy, hepatotoxicity and nephritis (Williams et al. 2003).

In spite of decades of efforts to unlock the secret of clozapine, it has not yet been possible to gain a better understanding of its superior efficacy. Several characteristics of the complex pharmacological profile of clozapine have been highlighted. For example, although the affinity for D_2 receptors is relatively weak, it may play a crucial role. Clozapine dissociates rapidly showing fast and transient dopamine D_2 receptor occupancy. The dopamine

system may become more sensitive with repeated transient blockade and therefore this property has been suggested as a reason for clozapine´s superior efficacy (Tauscher et al. 2004, Remington 2003, Kapur and Seeman 2001). Moreover, clozapine´s efficacy could be attributed to its affinity to bind to several other neurotransmitter receptors. These include D_4 receptors, the potent serotonin 5-HT_{2A} receptor antagonism, alterations of noradrenergic biochemistry and equivalent or higher occupancy of D_1 to D_2 receptors (Tauscher et al. 2004, Remington 2003, Horacek et al. 2006). Clozapine´s stimulation of the dopamine D_1 receptor in the medial prefrontal cortex may induce synaptic plasticity and is a further aspect of the atypical profile of the drug (Matsumoto et al. 2008). The regional distribution of the dopaminergic effect and serotonergic modulation through other monoaminergic receptors such as serotonergic 5-$HT2_A$, 5-$HT1_A$, and 5-HT_{2C} receptors may add to higher dopamine output in the striatum and prefrontal cortex (Horacek et al. 2006, Xiberas et al. 2001). This could also explain not only its efficacy with regard to cognitive symptoms but also possibly negative symptoms (Elsworth et al. 2008, Ichikawa et al. 2005). Furthermore, it has been suggested that the main metabolite of clozapine, *N*-desmethylclozapine, which achieves average plasma concentrations of 60 to 80% of that of clozapine, displays antipsychotic activity as a partial agonist of muscarinergic M_1 receptors, and of dopaminergic D_2 and D_3 receptors (Burstein et al. 2005).

A number of recent studies have investigated the expression of genes in animal models after clozapine treatment. These have used microarray technology to profile transcripts in brain tissue and revealed changes in genes involved in neurotransmission, signaling, neuronal and glial cell development and function, transcription factors, and enzymatic regulators, in multiple schizophrenia-associated brain regions (Duncan et al. 2008).

Clozapine may correct altered nuclear epigenetic functions. It was recently shown in an animal model that clozapine can induce cortical and striatal DNA demethylation and may therefore normalize GABAergic gene expression that is down-regulated in schizophrenia patients (Dong et al. 2008).

Animal studies and clinical observation in patients indicate that both FGAs and SGAs may influence synaptic plasticity. The second-generation antipsychotics and clozapine in particular may induce neuronal plasticity and

synaptic remodeling not only in striatum but also in other brain areas (Goff and Coyle 2001, Horacek et al. 2006, Matsumoto et al. 2008). They may also induce or upregulate transcript and protein levels of several neurotrophins involved in neuron rescue (de Bartolomeis et al. 2005), such as nerve growth factors (NGF) or brain-derived neurotophic factors (BDNF) (Pillai et al. 2006, Buckley et al. 2007). The results, however, are inconsistent and may depend on the duration of drug administration (Pillai et al. 2006, Terry and Mahadik 2007). Both increased and decreased levels of neurotrophins have been reported in rat brain and in patient serum following clozapine administration (Buckley et al. 2007, Lipska et al. 2001, Bai et al. 2003, Pirildar et al. 2004). An increase in apolipoprotein D that has been reported following treatment with clozapine, risperidone and olanzapine may also contribute to the neuroprotective effects (Mahadik et al. 2002, Thomas and Yao 2007).

There is some evidence that antipsychotic drugs may induce neurogenesis (Luo et al. 2005, Green et al. 2006). However, the evidence for clozapine is not conclusive (Schmitt et al. 2004, Halim et al. 2004). It is important to note that neurogenesis is not specific to antipsychotics but is also seen in response to a variety of other treatments such as chronic antidepressant treatment and electroconvulsive therapy (Buckley et al. 2007). Recent studies in patients with schizophrenia suggest, however, that proliferation of neural cells in the dentate gyrus is decreased in these patients. Clozapine may prevent this impairment (Maeda et al. 2007), as well as attenuate the loss of gray matter during the course of schizophrenia (van Haren et al. 2007).

Clozapine, together with some other antipsychotic drugs, has been shown to have immunomodulatory effects (Pae et al. 2006, Pollmacher et al. 2000). Given that during antipsychotic therapy there is evidence of immune alterations in schizophrenia and of activation of the adaptive immune systems, it is tempting to speculate that this could at least partly explain the efficacy of these drugs (Sperner-Unterweger 2005, Muller et al. 2000). Clozapine has been reported to alter the levels of several cytokines *in vivo* and *in vitro* and to induce signs of immune-activation during short-term treatment (Maes et al. 1997, Song et al. 2000, Maes et al. 2002, Rudolf et al. 2002). These findings have, however, been inconsistent. Following clozapine administration, schizophrenia patients have been reported to exhibit not only decreased levels of B-lymphocytes, increased levels of sIL-

2R (soluble interleukin-2 receptor) that inactivates IL-2 and altered levels of IL-6, but also both increased and decreased levels of TNF-α (tumor necrosis factor) and sTNF-R p55 and p75 (soluble tumor necrosis factor receptors). Conflicting results with regard to other cytokines include decreased production of lymphotoxin and altered levels of IL-1RA (interleukin-1 receptor antagonist), IL-18, IL-10, TGF-β (transforming growth factor), IFN-γ (interferon), and IL-4 (Drzyzga et al. 2006, McAllister et al. 1989). It has been proposed that clozapine-induced fever, which affects up to 50% of patients, could be linked to a transient IL-6 increase, a unique characteristic of clozapine when compared with other antipsychotics which are also known to activate the cytokine system (Kluge et al. 2009).

Considering the possible role of autoimmunity in schizophrenia, the immunomodulatory potency of clozapine could also contribute to the unique efficacy of the compound. Clozapine-induced agranulocytosis during treatment, together with some other findings such as decreased B-lymphocyte count or transient fever, could indicate an immunosuppressive effect (Goldsmith and Rogers 2008, Kluge et al. 2009).

4.3 Clozapine-induced agranulocytosis

The development of agranulocytosis (absolute neutrophil count <0.5 x 10^9/l) is associated with clozapine in some patients and may be independent of dosage (Pirmohamed and Park 1997, Hasegawa et al. 1994). Epidemiological studies have revealed that the incidence of clozapine-induced neutropenia is approximately 3%, whereas the incidence of agranulocytosis varies between 0.05 and 2% (Alvir et al. 1993, Abt et al. 1992, Gerson 1993) and is currently estimated at 0.8% (Dettling et al. 2007, Andres and Maloisel 2008). The onset of agranulocytosis is delayed, occurring usually 6-12 weeks after exposure to clozapine (Dettling et al. 2007). The risk is highest during the first 3 months of treatment, with 95% of cases occurring within the first 6 months (Iqbal et al. 2003) and decreasing exponentially over time (Schulte 2006). However, some cases are also reported after several years of continued therapy (Patel et al. 2002). Therefore, European monitoring routines require that patients on clozapine undergo weekly white blood cell monitoring for the first 18 weeks of therapy, decreasing thereafter to monthly monitoring for the remaining duration of clozapine administration (Wagstaff and Bryson 1995, Schulte

2006). Clozapine-induced agranulocytosis is usually characterized by a gradual decrease in white blood cell counts over several weeks (Patel et al. 2002), but in some patients this condition develops rapidly over a matter of days (Gerson and Meltzer 1992). The neutrophil count can increase significantly before decreasing (Uetrecht 1996). As the risk of death is 3-4%, agranulocytosis is considered to be a medical emergency (Schulte 2006). After the drug is withdrawn, the condition is treated with broad-spectrum antibiotics and hematopoietic growth factors (granulocyte colony-stimulating factor, G-CSF and granulocyte-macrophage colony-stimulating factor, GM-CSF) (Andres and Maloisel 2008, Schulte 2006) which may shorten the duration and reduce infectious and fatal complications (Andersohn et al. 2007). G-CSF may enhance the production of myeloid cells and their mobilization from the bone marrow (Kawai et al. 2007). Agranulocytosis is usually reversible within 14–22 days of discontinuation of clozapine therapy (Patel et al. 2002).

There are several risk factors associated with the development of agranulocytosis. Risk may increase with age and may be higher in women (Iqbal et al. 2003, Lieberman and Alvir 1992). Links between human leukocyte-antigen (HLA)-haplotypes and clozapine-induced agranulocytosis have also been reported, suggesting genetic susceptibility (Dettling et al. 2007, Lieberman et al. 1990). A further risk factor is concomitant treatment with other drugs known to induce agranulocytosis (Patel et al. 2002).

Neutropenia is defined as a neutrophil count of $<1.5L^{-9}$. The risk of developing neutropenia as a result of clozapine treatment varies between 0.9 and 2.9% (Lambertenghi Deliliers 2000, Kang et al. 2006, Munro et al. 1999, Atkin et al. 1996). Transient neutropenia (2-5 days) and weekly variations of neutrophil count are quite common during clozapine treatment and do not necessitate discontinuation of the drug (Flanagan and Dunk 2008). One report indicated that 22% of patients given clozapine for the first time had temporary granulocytopenia, but recovered within 2 weeks as drug treatment was continued (Hummer et al. 1994). Some patients with neutropenia may therefore recover spontaneously, despite continued treatment (Schulte 2006). Why some patients develop transient neutropenia whilst others progress to agranulocytosis is not known. According to epidemiological data, clozapine-induced neutropenia and agranulocytosis may be distinct disorders with different etiological mechanisms (Flanagan and Dunk 2008).

4.4. Mechanisms of clozapine-induced agranulocytosis

The exact mechanism of clozapine-induced of agranulocytosis is unclear. At therapeutic drug concentrations (1-3 µM), neither clozapine nor its major stable metabolites, *N*-desmethylclozapine or clozapine-*N*-oxide, have been found to be directly cytotoxic to neutrophils or interfere with the turnover of bone marrow precursor cells (Pirmohamed and Park 1997, Williams et al. 2000, Mosyagin et al. 2004). Also the delayed onset of agranulocytosis and the inability to reproduce the same reaction in animals speaks against simple toxic mechanisms (Dettling et al. 2007, Guest and Uetrecht 1999). An immune-mediated mechanism, or at least an immunological background for clozapine-induced agranulocytosis is feasible, as on re-exposure to the drug, the course is more severe and the time interval to recurrence of toxicity is shorter, suggesting an anamnestic response (Dunk et al. 2006). This time interval, however, is longer (6-12 weeks) than that usually observed with immune-mediated reactions (at most 2 weeks) (Pirmohamed and Park 1997, Guest and Uetrecht 1999). More direct evidence, such as the presence of antidrug or antineutrophil antibodies, has not been found (Pirmohamed and Park 1997).

Most of the drugs associated with a high incidence of agranulocytosis have been shown to be oxidized to reactive metabolites by the myeloperoxidase - hydrogen peroxide system of neutrophils that generates hypochlorous acid during oxidative bursts (Guest and Uetrecht 1999).

In vivo, clozapine is probably metabolized by the hepatic P450 enzymes, myeloid cells and peripheral blood polymorphonuclear leukocytes (Williams et al. 2003, Williams et al. 1997). As clozapine has been shown to have immunomodulatory effects resulting in changes in cytokine plasma levels, cytokines such as TNF-α could activate neutrophils or their precursors (Pollmacher et al. 1996). Activated neutrophils release myeloperoxidase (MPO) and hydrogen peroxide, which can oxidize (bioactivate) clozapine at therapeutic concentrations to a reactive intermediate, the nitrenium ion (Iverson et al. 2002). Reactive metabolites of clozapine can induce oxidative stress, which peripheral neutrophils and their bone marrow precursors are particularly susceptible to. It has also been shown that the nitrenium ion is capable of covalently binding to neutrophils and to proteins

in bone marrow tissue *in vitro* and *in vivo* (Williams et al. 2000, Maggs et al. 1995, Gardner et al. 1998, Park et al. 2000). When therapeutic concentrations of radiolabeled clozapine were used, up to 7% of the drug became irreversibly bound to neutrophils (Liu and Uetrecht 1995). *In vitro, a* protein-reactive metabolite of clozapine appears to accelerate neutrophil apoptosis through depletion of intracellular ATP and reduced glutathione (Andres and Maloisel 2008, Park et al. 2000, Husain et al. 2006).

It has been postulated that reactive metabolites of clozapine while covalently binding to macromolecules, could chemically modify critical proteins within the neutrophils or their precursors. They could be directly toxic, or alternatively, cause hapten formation and immune-mediated toxicity or hypersensitivity. Nevertheless, it is possible that there is more than just one mechanism for clozapine-induced agranulocytosis and neutropenia (Uetrecht 1996, Gardner et al. 2005).

Clozapine only induces agranulocytosis in few patients. If this is due to an immune reaction triggered by clozapine-modified polypeptides, only certain susceptible individuals could be affected (Gardner et al. 1998). Furthermore, individuals could possess varied potential to build effective defence mechanisms against oxidative stress, which could be related to age, diet, enzyme induction or genetic factors (Pereira and Dean 2006). For example, genetic variations related to the metabolization of neutrophil enzymes such as to the MPO gene resulting in reduced expression of MPO could theoretically have some relevance (Opgen-Rhein and Dettling 2008). As clozapine-induced agranulocytosis has been associated with various polymorphisms in the TNF-α gene, differences in individual TNF-α secretion could contribute to susceptibility to agranulocytosis (Williams et al. 1997). Moreover, HSP70 (heat shock protein) and NQO2 (dihydronicotinamide riboside quinone oxidoreductase 2) (Ostrousky et al. 2003, Corzo et al. 1995) have been associated with clozapine-induced agranulocytosis and as superoxide scavengers they may have a function in detoxification of clozapine. An individual's ability to secrete antiapoptotic cytokines could also contribute to the risk of agranulocytosis (Fehsel et al. 2005). A link between the pharmacological properties of the drug and its ability to induce agranulocytosis could be the finding that the drug or its reactive metabolites may target the neurotransmitter receptors found on stromal cells of the bone marrow. Those receptors are capable of modulating or impairing the function of stromal cells (Pereira et al. 2003).

4.5. Human leukocyte antigens and clozapine-induced agranulocytosis

The human leukocyte antigen (HLA) region or the human major histocompatibility complex (MHC) is located on the short arm of chromosome 6 at 6p21.3. This area encompasses about 250 genes (www.sanger.ac.uk; accessed 01.02.08) which have functions related to the immune system. The MHC is traditionally divided into the class I, class II, and class III regions. The class I region contains three main functional class loci, HLA-A, HLA-B, and HLA-C, all of which are highly polymorphic. The class II region includes HLA-DR, DQ and DP, while the class III region contains genes encoding the complement components, the heat shock protein (HSP70) family and the cytokine tumor necrosis factors (TNF), lymphotoxin A and B. It is typical for the HLA alleles to be in linkage disequilibrium, i.e. non-randomly associated at linked loci. This phenomenon may offer a selective advantage to its bearers.

Class I antigens are present on all nucleated cells, whereas class II molecules are particularly expressed on B-cells, dendritic cells and macrophages. In general, T lymphocytes are responsible for the specific recognition of pathogens and antigens, recognizing them on the surface of a cell only when the antigens are associated with HLA molecules. As a consequence of this recognition, lymphocytes activate and, depending on their subtype, contribute to the generation of either cell-mediated or humoral immunity by releasing a variety of cytokines; including a cascade of reactions leading to an immune response (Peh et al. 2000, Lahdelma and Koskimies 2004).

HLA class I and II are associated with several immune- and non-immune-mediated diseases, as well as adverse drug reactions (Dettling et al. 2007, Milner et al. 2000, Wright et al. 2000, Yunis et al. 1995). Susceptibility and resistance to almost all autoimmune diseases is associated with genes within the MHC. Classical examples include ankylosing spondylitis, Goodpasture's syndrome, dermatitis herpetiformis, insulin-dependent diabetes mellitus, multiple sclerosis and rheumatoid arthritis. The exact

mechanisms by which the HLA associations contribute to autoimmune disease susceptibility is still unclear. Possible explanations include repertoire differences through positive and negative selection on different class II genes, or preferential binding of disease-inducing epitopes of bacteria or viruses to particular HLA molecules, coupled to the influence of environmental factors (Cooke 2001, Hammond et al. 1988). Given that strong linkage disequilibrium is characteristic to the MHC region, a susceptibility gene may also be another MHC or non-MHC gene lying within the MHC and in linkage equilibrium, thus accounting for disease susceptibility. Class III antigens such as TNF and HSP may also play an important role in the immune response. Furthermore, non-MHC genes have been associated with MHC-linked diseases and non-MHC genes may influence immune response while encoding cytokine receptors or macrophage function (Warrens and Lechler 2000). Non-MHC genes, such as polymorphisms in the NQO2 gene at Chr 6p25, have been associated with HLA-B38, suggesting linkage equilibrium. Defective oxidative mechanisms linked to the NQO2 gene could lead to insufficient detoxification of reactive clozapine metabolites, resulting in neutrophil apoptosis (Opgen-Rhein and Dettling 2008).

In some HLA-associated diseases there is little or no evidence for a primary immunological process. For instance, narcolepsy is strongly linked with DQB1*0602 (Nishino 2007). Several adverse drug reactions have also been associated with HLA. Carbamazine-induced hypersensitivity has been associated with DR2 and DQ2, metamizole-induced agranulocytosis with A24, B7 and DQ1, penicilinamine-induced toxicity with DR4, and sulfonamide-induced toxic necrolysis with A29, B12 and DR7. However, most of these studies have not been replicated (Dettling et al. 2007).

Clozapine-induced agranulocytosis has not only been associated with both HLA class I and II, but also with class III genes for HSP and TNF (Dettling et al. 2007, Husain et al. 2006). Associations have also been found with HLA-B16, B38, DR4, DR2 and DQ1 (Yunis et al. 1995, Pfister et al. 1992, Joseph et al. 1992, Valevski et al. 1998) in both Jewish and non-Jewish Caucasian schizophrenic patients. More recent studies in Caucasian patients have found associations of agranulocytosis with HLA-Cw*7, DQB*0502, DRB1*0101, and DRB3*0202 (Dettling et al. 2007, Dettling et al. 2001), while a haplotype analysis of HLA classes I and II indicated a significant association with the two-locus haplotypes HLA-Cw-B and HLA-DRB5-DRB4,

and with the three-locus haplotypes HLA-Cw-B-DRB5 (Dettling et al. 2007). These reported HLA associations may point to an immuno-mediated mechanism behind clozapine-induced agranulocytosis, but other mechanisms such as aberrations in enzymatic pathways encoded by genes in linkage equilibrium to the HLA region must also be considered.

4.6. The association of human leukocyte antigens with schizophrenia and antipsychotic drug response

Data do not present strong evidence in favour of an HLA association with schizophrenia. Nevertheless, associations with HLA-A9, A10, and A28 have been reported (Debnath and Chaudhuri 2006). In a critical review, only evidence for associations with HLA DQB1*0602 and DRB1 *04 was found, as these may protect against schizophrenia (Wright et al. 2001). Interestingly, a recent genome-wide association study implicated the major histocompatibility complex on chromosome 6p. However, it was not possible to ascribe the association to a specific HLA allele, haplotype or region (International Schizophrenia Consortium et al. 2009)

HLA-G, a non-classical class I antigen, has been suggested to play a pivotal role in schizophrenia (Debnath and Chaudhuri 2006). In contrast to the classical HLA class I antigens, HLA-G is not expressed ubiquitously but by placental trophoblast cells. HLA-G probably has an important function in immune suppression at the maternal-fetal interface (Kuroki and Maenaka 2007). HLA-G antigens are prominent in the first-trimester of pregnancy, and reduced in the third trimester. Notably, maternal respiratory infections in the second trimester increase the risk of schizophrenia in offspring (Mednick et al. 1988). This has led to a hypothesis that maternal infections during this period may activate and direct both inflammatory and cytolytic components of the maternal immune system against developing neuronal tissue (Debnath and Chaudhuri 2006).

Evidence supporting the theory that several infections and inflammatory biomarkers may contribute to the etiology of schizophrenia is increasing (Brown 2006, Saetre et al. 2007). HLA-G is purported to play a vital role in the regulation of production of certain cytokines by the immune cells in the uterus. Although hypothetical, maternal infections could disturb the uterine immune milieu and lead to disturbance of HLA-G expression. This would

lead to the loss of HLA-G-mediated neuroprotection, resulting in abnormalities in brain development. In addition, selective linkage disequilibrium exists between HLA-A and HLA-G, which is of interest when considering the reported associations between schizophrenia and HLA-A antigens (Debnath and Chaudhuri 2006, Ober et al. 1996).

Drug-induced side-effects and response to antipsychotic drugs have been associated with specific HLA alleles in some studies. Studies on associations with extrapyramidal movement disorders have been inconsistent (Wright et al. 2000). **Table 1** summarizes studies reporting associations between antipsychotic drug response and HLA (Smeraldi et al. 1976, Bersani et al. 1989, Alexander et al. 1990, Meged et al. 1999, Marchini et al. 2001). Refractoriness criteria refer to criteria used to define resistance to neuroleptic treatment.

Table 1

HLA association studies on antipsychotic drug response in schizophrenia

Investigator	Drugs	Response Criteria	Refractoriness Criteria	Ethnicity	Patients (n) Diagnosis	Diagnostic criteria	Comparison subjects	Result
Smeraldi et al., 1976	a) Chlorpromazine, haloperidol, conventional neuroleptics b) Chlorpromazine	Clinical judgement, WPRS	-	Caucasian	a) Schizophrenia (n=33) 20 responsive and 13 non-responsive to standard neuroleptics b) Schizophrenia (n=17)	WHO classification	Population (n=386)	HLA-A1 correlated positively and HLA-A2 negatively to chlorpromazine responsiveness (values not corrected)
Bersani et al., 1989	Chlorpromazine, haloperidol	BPRS	-	Caucasian	Schizophrenia (n=91)	Research Diagnostic Criteria	Blood donors (n=321)	No association in the overall group HLA-A1 negative and HLA-A2- positive responsive in the paranoid subgroup. (A trend for increased frequency of HLA-A1 in non-responsive hebephrenic subgroup.)
Alexander et al., 1990	Haloperidol	BPRS	-	Caucasian	Schizophrenia (n=26)	DSM-III	Population (n=1029)	No association A trend for increased frequency of HLA-A11 in responsive group and for decreased frequency of HLA-A1 in responsive group after 1 week
Meged et al., 1999	Clozapine, haloperidol	Clinical judgement or CGI	Modified Criteria of Kane	Jewish	Schizophrenia or schizoaffective disorder (n=88) 38 responsive and 50 refractory to standard neuroleptics	DSM-III-R	Ethnically matched healthy controls (n=127)	No association A trend for elevated rates of HLA-B38 among controls and patients of Ashkenazi origin who were resistant to standard neuroleptics
Marchini et al., 2001	Clozapine	BPRS	Criteria of Kane	Caucasian	Schizophrenia (n=31) Refractory to neuroleptic treatment	DSM-IV	Matched healthy controls	HLA-B35 and HLA-A2 correlated positively to clozapine responsiveness (values not corrected?)

Over he past decades there are a limited number of studies and the methodological shortcomings in many of them make conclusions difficult. In earlier studies a technically less specific HLA serotyping was used. Sample sizes were small; the studies employed different criteria for diagnosis and drug response. The populations also varied, having diverse genetic backgrounds. Furthermore, the studies have no standards for drug treatment, therefore the drug dose or treatment duration might not have been sufficient to achieve an antipsychotic response. Statistical problems may also have biased the results, and corrected values were not always used. Nonetheless, the studies indicated a correlation with HLA-A1 and response to FGA treatment in patients with schizophrenia (Smeraldi et al. 1976, Bersani et al. 1989, Alexander et al. 1990). The presence of HLA-A1 may predict a poorer outcome to treatment with conventional antipsychotic drugs (Lahdelma and Koskimies 2004).

4.7 Clozapine-induced agranulocytosis and gene-expression

Gene expression is a complex and tightly regulated process that allows a cell to respond dynamically to environmental stimuli and its changing needs. This mechanism enables the cell to control which genes are expressed and to regulate the level of expression of each gene. Gene expression can therefore be decreased (down-regulated) or increased (up-regulated). Disruptions or changes in gene expression are responsible for many diseases.

Microarrays provide a way of analyzing expression of many hundreds of thousands of genes quickly and efficiently. A microarray works by exploiting the ability of a given mRNA (messenger RNA) molecule to hybridize or bind specifically to the DNA template from which it originates. Using an array containing many DNA samples, the expression levels of thousands of genes within a cell can be determined by measuring the amount of mRNA bound to each site on the array. With the aid of a computer, the amount of mRNA bound to the spots on the microarray is precisely measured, generating a profile of gene expression in the cell (www.ncbi.nlm.nih.gov/ About/primer/ microarrays. htlm, accessed 30.03.07).

Gene expression profiling during drug treatment holds promise for identifying gene expression changes that occur downstream of receptor

blockade and thus reveal new molecular mechanisms. Alterations in gene expression have also been reported in response to antipsychotic medications, and particularly to clozapine (Thomas 2006, Kontkanen et al. 2002b, Takahashi et al. 2004, Verma et al. 2007). The challenge, however, remains in identifying which changes are the most relevant. There is evidence to suggest that the clinical effects of antipsychotic treatment are the result of changes in gene expression (Thomas 2006). The latency of clinical efficacy after drug administration may result from a cascade of gene expression changes that gradually compensate for underlying neurochemical deficits. This is supported by reports of activation of immediate early genes after antipsychotic drug exposure (Thomas 2006, Kontkanen et al. 2002a). Side-effects after long term drug treatment, such as tardive dyskinesia may also result from changes in gene expression (Thomas 2006). Moreover, changes in gene expression may account for clozapine-induced side-effects, such as weight-gain and lipid abnormalities (Thomas 2006, Mehler-Wex et al. 2006).

Clozapine-induced agranulocytosis has been associated with gene expression alterations. A significant increase in FasL (Fas ligand) gene expression in neutrophils and HL-60 cells (human promyelocytic cells that are used as a cell culture model for human leukocytes) and decreased CD16 cell membrane expression was observed in the study of Husain et al. following clozapine bioactivation after 6 hours in a granulocyte culture (Husain et al. 2006). Both CD16 and FasL are cell surface associated proteins, which have been implicated in the apoptotic process. CD16 is a granulocyte membrane receptor for IgG antibodies and is linked with neutrophil apoptosis and antibody-dependent cellular cytotoxicity. FasL is a protein of the TNF family and its interaction with Fas–membrane protein may be necessary for triggering apoptosis in granulocytes. It has been suggested that the increased expression of FasL in clozapine treated cells accounts for increased apoptosis in these cells and is linked to generation of oxidized clozapine. As clozapine-induced agranulocytosis has been associated with HLA class III genes for TNF and HSP, the investigators proposed that the formation of oxidized clozapine intermediates may decrease the survival of granulocytes in individuals who carry clozapine-induced agranulocytosis susceptibility-associated HSP or TNF variants.

Gene expression has also been studied *in vivo* in blood cells of clozapine treated patients under long-term therapy with and without agranulocytosis.

Increased neutrophil apoptosis in all clozapine-treated patients was attributed to oxidative stress and increased expression of proapoptotic genes *bax α*, *p53*, and *bik*. These findings speak for oxidative mitochondrial stress in neutrophils of clozapine-treated patients and suggest that free radicals and oxidative stress may up-regulate proapoptotic genes contributing to the induction of apoptosis and clozapine-induced agranulocytosis (Fehsel et al. 2005).

4.8 Clozapine-induced agranulocytosis and stromal cells

Most research regarding the mechanisms of clozapine-induced blood cell dyscrasia has focused on the effects on various hematopoietic cell lineages. Some of the toxicity, however, may be directed at components of the bone marrow microenviroment, like the marrow stromal cells (Guest and Uetrecht 1999). Stromal or reticular bone marrow cells form a cellular network that extends into hematopoetic tissue and supports hematopoiesis. They produce hematopoietic growth factors and cytokines that regulate the production of blood cells. The stromal cells and extracellular matrix they produce establish the milieu in which hematopoietic precursor cells develop. Specialized niches within the bone marrow environment promote lineage-restricted pathways of cell maturation which also harbour myeloid precursor cells. A recent report indicated that bioactivated clozapine induced cytotoxicity in immortalized stromal cell lines suggesting that clozapine bioactivation may impair stromal cell function and thereby arrest granulopoiesis (Pereira and Dean 2006).

5. AIMS OF THE STUDY

The general aim of the study was to identify biological mechanisms which contribute to clozapine-induced agranulocytosis and clinical drug response. The specific aims of the present thesis were:

I To perform an HLA association study on two particular patient groups with schizophrenia defined by the responsiveness to clozapine or first-generation antipsychotic drug therapy (conventional neuroleptic treatment).

II Based on the findings in the previous study, to examine whether patients with schizophrenia carrying HLA-A1 allele were at risk of developing clozapine-induced agranulocytosis.

III To investigate the effect of clozapine on gene expression in the leukocytes of patients with schizophrenia who started clozapine treatment for the first time and to compare changes in gene expression in HL-60 cells before and after exposure to clozapine.

IV To investigate the effect of clozapine and bioactivated clozapine on primary cultures of human bone marrow mesenchymal stromal cells.

6. Subjects and methods

The nature of the studies was fully explained to all the study subjects and informed consent was obtained from each subject for all the research procedures carried out in studies. The study protocols were approved by the Ethics Committees of the hospitals or organizations involved: the Department of Psychiatry, Helsinki City Hospital; the National Public Health Institute; Kellokoski Hospital, Hospital District of Helsinki and Uusimaa; Aurora Hospital, City of Helsinki Health Care Center; and Helsinki University Central Hospital.

6.1 Subjects and methods in study I

The study subjects were Finnish-born in- and outpatients referred to the Department of Psychiatry, Helsinki City Hospital and who met diagnostic criteria for schizophrenia (DSM-III-R, Diagnostic and Statistical Manual of Mental Disorders, Third Edition, Revised, 1987). The patients were divided into two groups based on their response to antipsychotic drug treatment in accordance with the criteria of Kane et al. (Kane et al. 1988). These criteria classify a patient as refractory to treatment when at least three periods of treatment with neuroleptic agents (from at least two different chemical classes) have been carried out in the preceding five years at dosages equivalent to or greater than 1000 mg/d of chlorpromazine for a period of six weeks, each without significant symptomatic relief, and 'without a period of good functioning' within the preceding five years. Severity of psychopathology was assessed using the Brief Psychiatric Rating Scale (BPRS). Improvement was defined as a 20% decrease in the BPRS total scale plus either a post-treatment Clinical Global Impressions (CGI) scale rating of mildly ill (≤3) or a post-treatment BPRS score of 35 or less. All assessments were performed before the blood samples for HLA typing were taken. The first group, resistant to treatment with conventional antipsychotic drugs (FGA) but responsive to clozapine, comprised 19 patients (8 women and 11 men). Their mean age was 36 years (SD=9). The second group, responsive to FGAs, consisted of 19 patients (10 women and 9 men), whose mean age was 38 years (SD=12). Finnish healthy blood donors (n=120) were used as controls. Stable doses of clozapine varied between 200 and 700mg a day for a period of at least 4 weeks and of FGAs

between 157 and 1700 mg chlorpromazine equivalents a day for at least 4 weeks.

Laboratory methods: HLA-A, B and DR typing of peripheral blood T and B cells was performed using the lymphocytotoxicity method with commercial typing trays (3x72-well trays) from Pel-Freez, Wisconsin, USA.

6.2 Subjects and methods in study II

The study subjects were Finnish-born in- and outpatients who met the criteria for schizophrenia according to DSM-III-R. Four subjects belonged to multiply affected families that were collected in an large genetic study. The subjects gave their informed consent after the procedure had been fully described to them. All of the subjects were receiving clozapine treatment and were divided into two groups based on their response. The first group consisted of 19 patients (8 women, 11 men; mean age 35.7 years [SD=9.2]) who exhibited a clinical response to clozapine and a lack of response to FGAs based on the criteria for treatment refractoriness used in study I. The mean dose of clozapine was 494mg (SD=160). The second group comprised 26 patients (15 women, 11 men; mean age 34.9 years [SD=12.5]) with a history of nonfatal clozapine-associated granulocytopenia (neutrophil granulocytes $<1.5 \times 10^9$/L) or agranulocytosis (neutrophil granulocytes $<0.5 \times 10^9$/L). Treatment had lasted 161.4 days (SD=342.5), with a mean dose of 405.8mg clozapine was (SD=153.2). Healthy Finnish blood donors (*n*=120) served as controls.

The patients were classified according to their type of neutropenia into mild-to-moderate type (absolute neutrophil count 0.5-1.5 x 10^9) or a more severe type (absolute neutrophil count $<0.5 \times 10^9$). Eight of the 26 patients, 6 women and 2 men, had severe neutropenia. The mean age in the group with severe neutropenia was 43.0 years (SD=10.6) and 34.9 years (SD=12.5) in the group as a whole.

Laboratory methods: HLA-A and –B typing was performed using fresh peripheral blood T cells by means of antibody-mediated lymphocytotoxicity using commercial typing trays (2 x 72 well trays) (Peel-Freez; Brown Deer,

Wisconsin, USA). In the agranulocytosis/granulocytopenia group, four patients were typed for the A locus and at the DNA level, using an HLA-A "low resolution" PCR-SSP typing kit (Dynal, Oslo, Norway).

6.3 Subjects and methods in study III

Eight Caucasian Finnish-born in- and outpatients (7 men and 1 woman; mean age 29.8 years; range 19-43 years) who met DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision, 2000) diagnostic criteria for schizophrenia and who responded insufficiently to other antipsychotic treatments were included in the study. None of the patients had previously been treated with clozapine. Exclusion criteria included the presence of other Axis I diagnoses besides schizophrenia, such as acute infectious or inflammatory responses for at least 2 weeks prior to the study, chronic medical illnesses, as well as any medication other than psychopharmacological treatment known to interfere with immune or endocrine function, such as treatment with corticosteroids. Patients with abnormal physical examination and abnormal blood and urine tests such as liver, renal or thyroid function tests were also excluded. Other psychotropic drugs received by patients concomitantly with clozapine included olanzapine (n=6), quetiapine (n=1), chlorpromazine (n=2), valproic acid (n=1), topiramate (n=1), sertraline (n=1), citalopram (n=2), diazepam (n=3), temazepam (n=1), zopiclone (n=4), and lorazepam (n=3).

Peripheral blood samples were collected both before starting clozapine treatment and during the treatment at time intervals of 0 hours, 3 hours, 3 days, 2 months and 4 months in order to investigate the effect of clozapine on gene expression in patient granulocytes.

Laboratory methods

Gene expression patterns were compared using cDNA (complementary DNA) microarrays on HL-60 cells (human promyelocytic leukemia cells) that were either treated or non-treated with clozapine. Subsequently, four candidate genes implicated in the maturation or apoptosis of granulocytes were selected and the alterations confirmed by QRT-PCR (quantitative real-

time reverse transcriptase-polymerase chain reaction). QRT-PCR was then performed on the patient samples collected at the different time intervals. The gene expression was also compared between mononuclear leukocytes and granulocytes.

HL-60 cells

The gene expression pattern was compared between clozapine-treated (30μM clozapine) and non-treated HL-60 cells. The HL-60 cells were cultured for three days in RPMI-1640 medium supplemented with 10% fetal bovine serum, with and without clozapine. RNA extraction for the array, cDNA hybridization and analysis methods were all performed as described below.

Total RNA preparation in patient samples

Blood sample preparation and RNA extraction were performed as previously described, but with minor modifications (Airla et al. 2004). Peripheral blood mononuclear and polymorphonuclear cells were separated from whole blood at each time interval by Ficoll-Hypaque (Sigma) density gradient centrifugation. Subsequently, separated cells were washed twice using PBS (Phosphate-Buffered Saline) and centrifuged at 1200 rpm for 10 minutes. The pellet was dissolved in RLT buffer (Qiagen, BmbH, Hilden, Germany). RNAs were extracted from the cells using a Qiagen RNA extraction kit. RNA concentration was measured using a spectrophotometer (Gene Quant Pro; Amersham Pharmacia Biotech, New Jersey, USA).

Array procedure

Atlas Human Hematology/Immunology Arrays (Clontech Laboratories, Inc, USA) were used to screen the samples for gene expression. Each filter contained 588 duplicate spots representing cDNAs of known and sequence-verified genes. These genes are known to be expressed in normal hematopoietic cells and hematological disorders. The cDNA spots were immobilized on a nylon membrane.

cDNA array hybridization

Total RNA (3-4µg) was converted into cDNA (complementary DNA) and labeled with ^{33}P-dATP (P-labeled deoxyadenosine 5'-triphosphate) using the Atlas pure total RNA labeling system (Clontech), with probes purified and hybridized according to the manufacturer's instructions. The arrays were exposed to an imaging plate (BAS-MP 2040S; Fuji, Kanagawa, Japan) for 3-7 days, followed by scanning of the plate with a phosphorimager (Bio-Imaging Analyzer, BAS-2500; Fuji). Autoradiographic intensity was analyzed using Atlas Image 1.5 software (Chlontech).

Quantitative real-time reverse transcriptase-polymerase chain reaction

(QRT-PCR)

QRT-PCR analysis was performed to confirm the biologically relevant altered gene expression patterns of four genes: *MPO* (myeloperoxidase precursor), *MNDA* (myeloid cell nuclear differentiation antigen), *FLT3LG* (Fms-related tyrosine kinase 3 ligand) and *ITGAL* (antigen CD11A, lymphocyte function-associated antigen1) in the leukocytes of clozapine-treated patients and in the HL-60 cells before and after exposure to clozapine. A total of 500ng RNA was converted into cDNA using The First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics Corp., Indianapolis, USA) and oligo-p$(dT)_{15}$ was used. RT-PCRs were performed simultaneously on all the samples in order to minimize variation in the amount and quality of cDNA between samples.

Gene-specific PCR primers designed for the analysis of selected genes were acquired from TIB Molbiol (Berlin, Germany) (**Table 2**).

Table 2

Sequence data of primers used in QRT-PCR (quantitative real-time reverse transcriptase / polymerase chain reaction)

Gene	Primer	Annealing Temperature
ITGAL	F; ggCTATACTTgTCTTgTTCAC R; gCAATggCgCAATCTT	62°C
MNDA	F; AAgAgCAgAgTAAgCCC R; ggAACATTTCTTCTTgCATC	65°C
MPO	F; CTgTTCTgggTgCAgC R; CCACAgAgCCAggATT	62°C
FLT3LG	F; ggCTCAAgACTgTCgC R; CgggAgATgTTggTCTg	65°C

As employed in a previous study (Aalto et al. 2001), PCRs were performed in a 10µl volume with 1µl 'Hot Start' reaction mix from the LightCycler-Faststart DNA Master SYBR Green I kit (Roche Diagnostics), 2.6 mM $MgCl_2$, 0.5 mM of each primer and 1 µl of diluted cDNA (1:5, 1:10 or 1:50).

The LightCycler run was started with an initial denaturation at 95°C for 7 min. The target DNA was amplified by performing 45 cycles of denaturation at 95°C for 15 sec, annealing at 58-66°C for 5 sec and elongation at 72°C for 10 sec, followed by 20 sec at 55°C and then slow heating of the samples to 95°C at the rate of 0.1°C/sec with continuous fluorescence detection.

Each sample was run in parallel with the reference sample. A negative control without cDNA was included in each run. In addition, standard curves for calculating the relative concentrations were obtained by running a dilution series of the β globulin gene (LightCycler-Control Kit DNA; Roche Diagnostics) in each assay according to the manufacturer's instructions. The Second Derivative Maximum method provided by the LightCycler software was used to calculate the concentration values for the PCR product of each sample. The concentration of each gene product was determined based on kinetic approach. Results are expressed as ratio value of expression, obtained by comparison of the concentration values of genes from each sample with the average value of expression from the reference sample.

The brightness of the hybridization images was reduced for the cDNA array analysis so that the level of background intensity was approximately the same in all images. The intensities of the gene spots were obtained for autoradiography using Atlas Image 1.5 software (Clontech). The intensity of each spot on the array was compared with the intensity of the corresponding spot in the reference array. Firstly, the background intensity of the hybridization image was subtracted from all intensity values. Then, the intensity of each gene spot in the reference array was subtracted from the corresponding intensity in the sample array, in order to get the intensity difference to represent the difference in the expression of a given gene between the sample and the reference. The data were globally normalized by subtracting the average of the intensity differences of the genes in one array from each intensity difference value. This standardized the sample average in all arrays to zero. In addition, the variance was standardized to one by dividing each of the normalized intensity differences of an array by the standard deviation.

6.4 Subjects and methods in study IV

Human bone marrow mesenchymal stromal cells (MSCs) were obtained from heparinezed bone marrow aspirates of five healthy male volunteer donors undergoing bone marrow harvest for sibling transplantation. Four of the donors were aged between 21 and 29, and the fifth was 72.

A previously described procedure was used to isolate the marrow stromal cells (Mandelin et al. 2006). In brief, the mononuclear cell fraction was isolated using density gradient centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden) (Mandelin et al. 2006). The cells were cultured in complete culture medium consisting of DMEM (Dulbecco's Modified Eagle's Medium) with low glucose (Sigma, St Louis, MO, USA), supplied with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Euroclone, Siziano, Italy), as well as 10% FCS (Fetal Calf Serum) (tested for ability to support mesenchymal stem cell expansion; StemCell Technologies, Vancouver, BC). Mononuclear cells were plated at a density of $4x10^5/cm^2$ and incubated under standard cell culture conditions (37°C and 5% CO_2). After three days, the adherent cells were washed thoroughly with PBS to remove nonadherent cells, and then cultured further in complete culture medium. Cells were passaged when approximately 80% confluent.

The stromal cells cultured in this way expressed CD29, CD44, CD105 and CD166, but not CD34, CD45 or CD14 (data not shown). The multilineage potential of the MSCs was assessed according to their ability to differentiate into osteoblasts and adipocytes as described (Pittenger et al. 1999). The expression of alkaline phosphatase and calcium deposition were used as markers for osteogenic differentiation, and positive staining of lipid droplets with Oil Red-O identified adipogenic differentiation. The cells showed both osteogenic and adipogenic potential (data not shown). The MSCs were cultured in DMEM (low glucose) supplemented with 10% FBS, glutamine, and penicillin (Haartman Institute, Helsinki, Finland). Prior to confluency, the cells were detached by treatment with trypsin (0.25%)–versene and passaged at a density of 5000 cells/cm^2. Cells from passages four to five were used for the experiments.

Primary human skin fibroblasts were cultured in RPMI-1640 medium supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. Passages eight and 17 were used for the experiments.

6.4.1 Clozapine and bioactivation of clozapine

Clozapine was obtained from Novartis Pharmaceuticals. It was dissolved in 0.1 N HCl at a concentration of 150mM and diluted to a concentration of 10 µM using DMEM-1640 medium. The MSCs and human fibroblasts were incubated with the drug at a concentration of 10 µM in the absence or presence of the oxidation system. A total of 3 U horse radish peroxidase (HRP)/ 20 000 cells in a total volume of 100 µl were added to each 96-well cell culture plate, and the reaction was started using 25µM H_2O_2, both diluted in PBS as described (Williams et al. 1997, Pereira and Dean 2006). Suitable concentrations of HRP and H_2O_2 for subsequent assays were determined in preliminary experiments (data not shown). The plates were incubated at 37ºC and 5% CO_2 in air for 24 h. The experiments were performed using 3-5 parallel wells per condition.

6.4.2 ATP Luciferase Assay

An ATP Luciferase Assay, which quantifies the amount of cellular ATP, was used to detect cytotoxicity of the cell cultures. The ATP concentration declines rapidly in cellular necrosis or apoptosis (Fan and Wood 2007, Cali et al. 2008). A total of 50µl ATPlite mammalian cell lysis solution (PerkinElmer) was added to each well in a plate containing 20 000 mesenchymal stromal cells or human fibroblasts in the absence or presence of bioactivation, with or without the drug. The plate was shaken for 5 min at 500rpm, after which 50µl substrate solution (Luciferase/Luciferin, PerkinElmer) was added to each well and then the plate was shaken again for a further 5 min at 500rpm. The reaction mixtures were transferred to a black 96-well plate and luminescence was measured using a Wallace Victor^2 1420 multilabel counter (PerkinElmer) or a Fluoroscan Ascent FL (Thermo Fisher Scientific**,** Waltham, MA, USA).

6.5 Statistical analyses

6.5.1 Statistical analysis in study I

A two-sided Fisher's exact test was used for comparisons of two proportions. The *P*-values were adjusted for multiple comparisons, with *P*-values<0.05 considered as statistically significant. *P*-values were corrected using the number of comparisons made (n=19). StatXact 3 for Windows

(1995) was used for statistical calculations. For differences of proportions, 99% confidence intervals were determined using the Confidence Interval Analysis program (Gardner et al. 1996). Relative risk was estimated as recommended by Svejgaard (Svejgaard et al. 1983).

6.5.2 Statistical analysis in study II

Fisher's exact test was used to test the statistical significance. *P* values were corrected using the number of comparisons made (n=23). *P*-values lower than 0.002 were considered as significant at the corrected level, and odds ratios (OR) were calculated.

6.5.3 Statistical analysis in study III

The data were exported to an Excel spreadsheet and statistical analysis made using StatSoft statistica (version 5.0). Following correlation analyses, two-tailed nonlinear equation model Student *t* tests were used to evaluate the fold changes in gene expression before and after treatment. Significance was defined as *P* less than 0.05.

6.5.4 Statistical analysis in study IV

In the study comparing MSCs and human fibroblasts the values given for stromal cells were the mean ±SD of four separate experiments and for human fibroblasts the mean ±SD of three separate experiments. The values comparing the effect of the bioactivation system were the mean ±SD of two separate experiments for MSCs and one experiment for human fibroblasts. The results of the individual experiments were combined and normalized to the values of the control MSCs and fibroblasts, which were taken as 100%. The differences in means were analyzed by means of the Student's two-tailed *t*-test (two-sample equal variance) and the statistical difference was compared using untreated cells as controls. $P<0.05$ was considered as significant.

7. RESULTS

7.1 Association between HLA and response to antipsychotic drug treatment (Study I)

Three HLA loci (HLA-A, -B, and –DR) were analyzed in two groups of patients who were categorized according to their response to antipsychotic drug treatment and the results compared to normal controls. The group consisting of patients resistant to treatment with FGAs (first-generation antipsychotics or conventional antipsychotics) but responsive to clozapine (n=19) differed significantly for one HLA allele compared with both the other group [patients responsive to FGAs (n=19)], and the controls. While the frequency of HLA-A1 in the Finnish normal population is 20%, 58% of the patient group not responding to FGAs but responding to clozapine carried HLA-A1. This figure was only 10.5% for the patients responsive to FGAs. This resulted in a difference of 37.5% between the patient groups, P=0.0011 (99% confidence interval 7.2-68.6%). However, when the P-value was corrected for the number of comparisons made (2x29), it did not remain significant (P=0.064). No other statistically significant differences were observed. A slight non-significant increase in the frequency of HLA-B8 allele was seen in clozapine treated patients, but it did not extend to the HLA-DR3 allele.

7.2 Association between HLA, antipsychotic drug response and clozapine-induced agranulocytosis (Study II)

Two HLA loci (HLA-A and HLA–B) were analyzed in two groups of patients, categorized according to their response to clozapine treatment. The results are shown in **Table 3**.

In the group with clozapine-induced agranulocytosis or granulocytopenia (n=26) the frequency of HLA-A1 was 11.5%, whereas the frequency of HLA-A1 in the group not responding to FGAs but responsive to clozapine (n=19) was 57.9% (p=0.0013). HLA-A1 occurres at a frequency of 20.1% in the healthy controls. The difference in HLA-A1 frequency between both patient groups was statistically significant (p= 0.0013). In addition, agranulocytosis

or granulocytopenia was found to be associated with A28 specificity. The frequency of A28 in the patients with agranulocytosis or granulocytopenia was 30.7%, compared with 5.3% in the responder group (p=0.027). However, the p-value was no longer significant after correction. Furthermore, a weak association between HLA-B16 and agranulocytosis was found. The frequency of the B16 allele was 27.3% in the agranulocytosis/granulocytopenia group, but only 9.3% in the healthy controls (p=0.027). The frequency of the B16 allele did not differ significantly in the two study groups. Moreover, HLA-B40 was expressed in nearly 23% of the patients who developed agranulocytosis or granulocytopenia but in none of the clozapine responders. Again, the p-value was no longer significant after correction.

Table 3

Distribution of human leukocyte antigen (HLA) alleles among patients with schizophrenia who have clozapine-induced agranulocytosis/granulocytopenia (*n*=22), patients refractory to FGAs (conventional antipsychotics) but responsive to clozapine (*n*=19) and controls (*n*=120)

HLA Allele	Odds Ratio	Granulocytopenia/ Agranulocytosis	Clozapine Responder	Control [b]
A1[a]	0.09	**11.5** (*p*=0.0013)	**57.9**	20.1
A2[a]		38.5	47.4	54
A3[a]		36.4	42.1	44.4
A9[a]		23.1	5.3	16.2
A10		7.7	5.3	7.8
A11[a]		19.2	5.3	8.8
A19[a]		15.3	15.8	19.6
A28[a]	8.0	**30.7** (*p*=0.027)	**5.3**	11.3
B5		22.7	15.8	11.9
B7		13.6	21.1	24.3
B8		22.7	31.6	20
B12		13.6	10.5	15.4
B13		9.1	5.3	6
B15		18.2	15.8	20.1
B16	3.2	**27.3** (p=0.027)[c]	10.5	**9.3**
B17		0	5.3	4.3
B18		4.5	10.5	10
B22		9.1	5.3	5
B27		4.5	15.8	14
B35		22.7	26.3	27.2
B37		4.5	10.5	1.6
B40		22.7	0	17.9

[a] *n*=26

[b] Random Finnish population

[c] Comparison to controls

All *p*-values are corrected.

None of the patients with severe neutropenia (absolute neutrophil count $<0.5 \times 10^9$) carried the HLA-A1 allele.

7.3 Gene expression alterations in leukocytes of clozapine-treated schizophrenic patients (Study III)

7.3.1 Gene expression profiling using a cDNA array

When comparing clozapine-treated with untreated HL-60 cells, gene expression profiling using a cDNA array showed an altered expression in 24 of the 588 genes analyzed (**Table 4**).

Table 4

Gene expression profiling using a cDNA array: list of genes with altered gene expression in HL-60 cells before and after exposure to clozapine (GDB = the Human Genome Database)

Over-Expressed Genes	**GDB Accession Number**
ITGB3 (Integrin, beta-2 (antigen CD18 (p95)	M15395
NPM1 (nucleophosmin (nucleolar phosphoprotein B23, numatrin)	M23613
GSTP1 (glutathione S-transferase pi)	M24485
CCL5 (T-cell specific protein p288)	M21121
CD58 (CD58 antigen, (lymphocyte-function-associated antigen 3)	Y00636
ITGAL (antigen CD11A (p180), lymphocyte-function-associated antigen 1)	Y00796
MNDA (myeloid cell nuclear differentiation antigen)	M81750
RAC2 (Ras-related C3 botulinum toxin substrate 2, Small G protein)	M64595
EV12B (Ecotropic viral integration site 2B protein)	M60830
FTH1 (ferritin, heavy polypeptide 1)	M97164
BP1 (bactericidal/ permeability-increasing protein)	J04739
Under-Expressed Genes	
MYC (Myc proto-oncogene protein)	V00568
ITGA2 (integrin alpha-2 precursor, Platelet membrane glycoprotein Ia)	X17033
XRCC1 (DNA repair protein XRCC1)	M36089
ABCC1 (multiple drug resistance protein 1)	L05628
NOTC1 (neurogenic locus notch homolog protein 1)	M73980
MLLT6 (Myeloid/lymphoid or mixed-lineage leukemia, translocated to, 6)	U07932
CX3CL1 (chemokine ligand 1, small inducible cytokine subfamily D)	U91835
TNFSF8 (CD30 ligand 2, tumor necrosis factor superfamily, member 8)	L09753
FLT3LG (Fms-related tyrosine kinase 3 ligand)	U03858
CD47 (leukocyte surface antigen CD47)	X69398
ACK1 (activated p21cdc42Hs kinase)	L13738
MPO (Myeloperoxidase precursor)	M19507
NK4 (natural killer cell transcript 4)	M59807

Eleven genes were over-expressed and thirteen under-expressed. Four of the genes were selected for quantitative RT-PCR analysis, all of which have

been implicated in the maturation or apoptosis of granulocytes. Two genes, *FLT3LG* (Fms-related tyrosine kinase 3 ligand) and *MPO* (myeloperoxidase precursor) showed down-regulated expression, while the other two, *MNDA* (myeloid cell nuclear differentiation antigen) and *ITGAL* (antigen CD11A, lymphocyte function-associated antigen1) were over-expressed after treatment with clozapine.

7.3.2 Quantitative RT-PCR for selected genes of HL-60 cells

Table 5 shows the gene expression patterns in a cDNA array and QRT-PCR in the HL-60 cells.

Table 5

Gene expression patterns in a cDNA (complementary DNA) array and QRT-PCR (quantitative real-time reverse polymerase chain reaction) in HL-60 cells.

Gene	Gene expression	
	cDNA Array	QRT-PCR
MPO	Under	Under
	0.179	0.036
MNDA	Over	Over
	2.116	5.526
FLT3LG	Under	Under
	0.182	0.606
ITGAL	Over	Under
	1.97	0.637

Comparison of QRT-PCR analysis and the cDNA array is shown before and after 3 days´ clozapine treatment (fold changes)

Whilst the other genes were validated, *ITGAL* was not confirmed by QRT-PCR. *MNDA* exhibited up-regulation, while both *MPO* and *FLT3LG* exhibited down-regulation.

7.3.3 Quantitative RT-PCR for selected genes in patient blood samples

Mononuclear leukocytes in patient samples

The expression profiles of the four selected genes from the mononuclear leukocytes in the clozapine-treated patients are shown in **Table 6a**. When pooled RNA from mononuclear leukocytes was analyzed, the *MPO* and *FLT3LG* genes showed a trend for down-regulation and *MNDA* for up-regulation (**Table 6b**). Only sufficient RNA could be extracted using pooled samples for the analysis of the expression profile after 2 and 4 months.

Table 6a

Expression pattern of selected genes from mononuclear leukocytes for individual patients with schizophrenia at 3 hours and 3 days after clozapine initiation (fold changes)

Mononuclear leukocytes Pt no.		*MPO*	*MNDA*	*FLT3LG*	*ITGAL*
1	3 h	0.991	1.116	0.8154	1.5832
	3 days	0.9645	0.987	0.6875	1.3337
2	3 h	0.9865	0.841	1.30259	0.5719
	3 days	0.9638	0.3941	0.99312	0.4084
3	3 h	0.9895	2.797	0.80501	
	3 days	0.9769	1.969	0.6992	
4	3 h	0.976	0.563	0.9146	1.272
	3 days	0.9656	1.032	0.7764	1.247
5	3 h	0.9406	1.055	0.84156	0.971
	3 days	0.97518	1.056	0.52967	0.976
6	3 h	0.9984	1.028	0.396	1.522
	3 days	0.9709	1.055	0.489	1.4408
7	3 h	1.015	1.214	0.8014	2.583
	3 days	1.0417	1.195	0.5985	2.8679
8	3 h	0.9839	1.047	0.8607	1.508
	3 days	0.9748	0.906	1.4894	0.943
P value 1-8 for 3 h		**0.0446**	0.2057	0.0556	0.0589
P value 1-8 for 3 days		**0.0281**	0.3214	0.7367	0.1549

Table 6b

Expression pattern of pooled RNA from mononuclear leukocytes for patients with schizophrenia at 3 hours, 3 days, 2 months and 4 months after clozapine initiation (fold changes)

Pooled RNA from mono-nuclear leukocytes	*MPO*	*MNDA*	*FLT3LG*	*ITGAL*
3 h	0.98	1.06	0.92	0.88
3 days	0.96	1.12	0.82	0.92
2 months	0.84	0.74	0.92	1.23
4 months	0.74	1.68	0.83	4.02
P value	0.061	0.249	0.194	0.009

Granulocytes in patient samples

Due to technical reasons it was not possible to extract sufficient RNA from granulocytes of individual patients to allow QRT-PCR analysis. Therefore, samples were pooled at each time point. **Table 7** illustrates the expression pattern of the selected genes.

Table 7

Expression pattern of pooled RNA from granulocytes for patients with schizophrenia at 3 hours, 3 days, 2 months and 4 months after clozapine initiation (fold changes)

Pooled RNA from granulo-cytes	*MPO*	*MNDA*	*FLT3LG*	*ITGAL*
3 h	0.76	1	0.967	1.007
3 days	0.599	1.037	0.662	0.947
2 months	0.521	1.044	0.602	0.939
4 months	0.5	2.85	0.82	6.46
P value	**0.0018**	**0.0002**	0.2010	0.0757

Three hours after drug intake the myeloperoxidase precursor gene was already significantly underexpressed and after 3 days the *MNDA* gene was overexpressed, resembling the kinetics of altered gene expression seen in the HL-60 cells. Furthermore, both genes remained either down-regulated or up-regulated at 2 months and 4 months respectively (Table 7). In addition, the *FLT3LG* gene showed linear down-regulation at all time points, but less significantly. By contrast, the *ITGAL* gene was up-regulated at 3 hours, down-regulated at 3 days and 2 months, and up-regulated again at 4 months.

7.4. Effect of clozapine on the primary cultures of human bone marrow stromal cells (Study IV)

Mesenchymal stromal cells and human fibroblasts incubated with clozapine

MSCs and fibroblasts were incubated with 10μM of clozapine for 24 hours and then cell viability was measured using an ATP Luciferase Assay to detect possible toxic effects of the clozapine. It was found that the MSCs were very sensitive ($p<0.05$) to the toxic effects of 10μM clozapine (**Figure 1**). Clozapine was not toxic to fibroblasts, appearing rather to stimulate their growth ($p=0.006$).

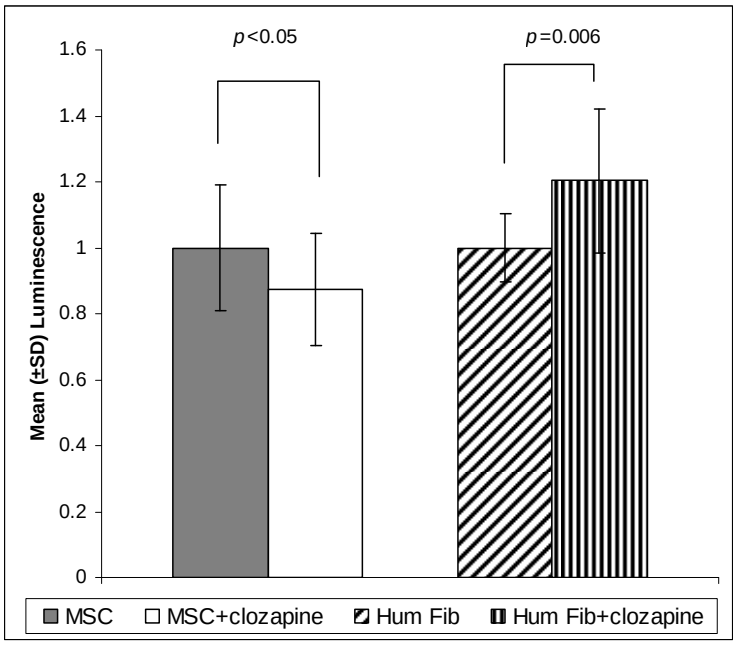

Figure 1. The effect of 10µM clozapine on cultures of mesenchymal stromal cells (MSC) and skin fibroblasts (Hum Fib)

The cells were treated for 24h and the ATP content measured using quantitative bioluminescence. The mean values ±SD are shown. The statistical differences between untreated cells and cells treated with clozapine are indicated.

Mesenchymal stromal cells and human fibroblasts incubated with clozapine with or without oxidative bioactivation

Earlier studies have indicated that clozapine toxicity may be aggravated by bioactivation. We therefore tested whether bioactivation of clozapine influences its toxic effect on primary MSCs. A previously reported oxidative bioactivation system for clozapine was used (Williams et al. 1997, Pereira and Dean 2006).

These experiments showed that while unmodified clozapine at a concentration of 10µM was toxic to MSCs, bioactivation with HRP+H_2O_2 nullified this toxicity (**Figure 2**). The difference was significant (p=0.006)

between untreated and clozapine-treated cells in the absence of bioactivation. Treatment of MSCs with the oxidation system alone did not induce cytotoxicity (p= 0.22). Interestingly, oxidation counteracted the toxicity of clozapine, since the difference between untreated cells and cells treated with bioactivated clozapine was not significant (p=0.50).

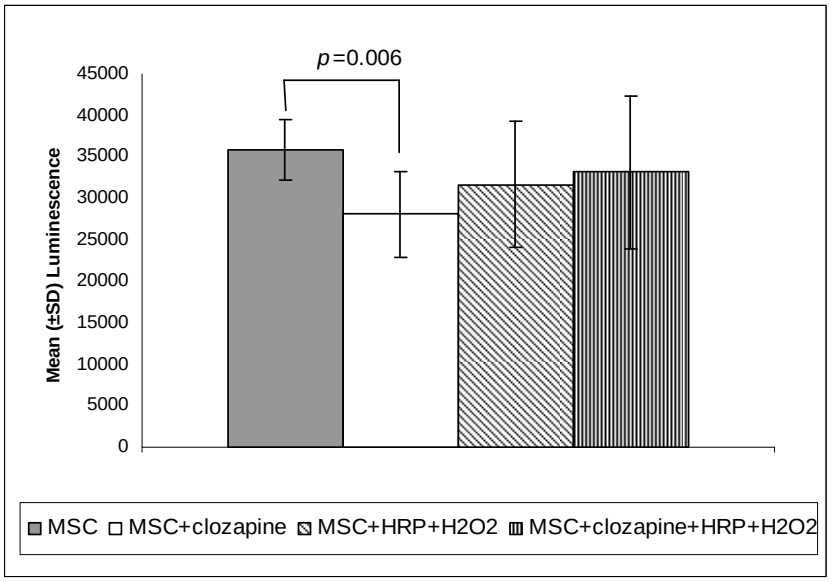

Figure 2. Effect of unmodified clozapine (10µM), the bioactivation system (HRP+H_2O_2) and bioactivated clozapine on the viability of MSCs

Mean luminescence values ± SD.

Clozapine (10µM) with or without bioactivation had no toxic effect on the fibroblasts (**Figure 3**). Clozapine without bioactivation had a growth-stimulating effect in comparison with control cultures (p=0.007). Bioactivation of clozapine seemed to cancel its growth-stimulating effects on fibroblasts, but the difference between clozapine-treated fibroblasts and fibroblasts treated with bioactivated clozapine was not significant (p= 0.08). Bioactivation alone stimulated fibroblast growth, but this effect nearly disappeared when combined with clozapine. The difference between fibroblasts treated with the bioactivation system and those treated with bioactivated clozapine was not significant (p=0.06).

These results show that clozapine is cytotoxic to MSCs without bioactivation but, in contrast, has a growth-stimulating effect on fibroblasts. These

effects of clozapine disappear when the drug is bioactivated by means of oxidation.

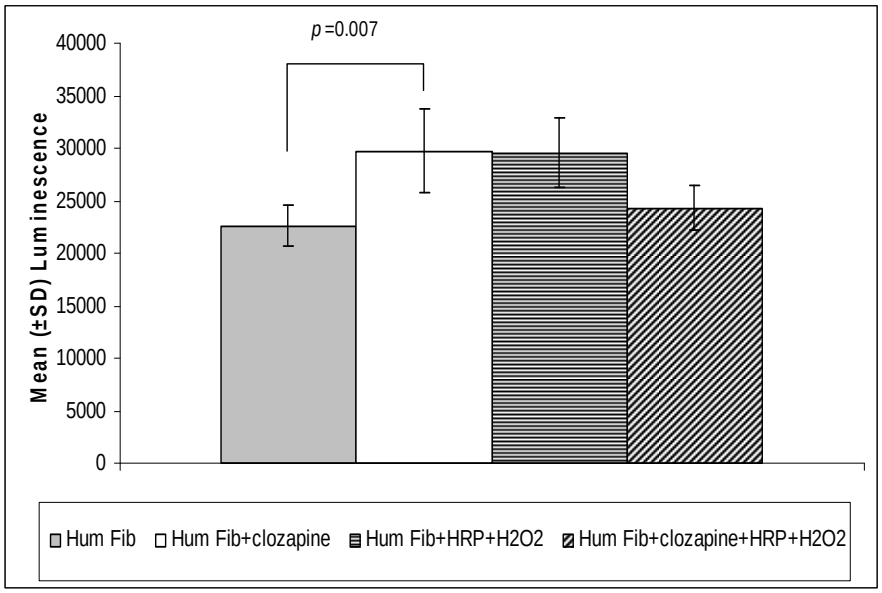

Figure 3. Effect of unmodified clozapine, the bioactivation system ($HRP+H_2O_2$) and bioactivated clozapine (10µM) on the viability of fibroblasts

Mean luminescence values ± SD.

8. DISCUSSION

8.1 Main results

The findings of our studies suggest new mechanisms behind clozapine-induced agranulocytosis and drug efficacy. The HLA-associations in schizophrenic patients with clozapine-induced agranulocytosis or in patients refractory to first-generation antipsychotics but responding to clozapine may suggest a genetic linkage in the Finnish population which contributes to these specific drug effects. We also identified gene expression alterations in 4 genes implicated in the maturation or apoptosis of granulocytes: MPO (myeloperoxidase precursor), MNDA (myeloid cell nuclear differentiation antigen), FLT3LG (Fms-related tyrosine kinase 3 ligand) and ITGAL (antigen CD11A, lymphocyte function-associated antigen 1) after clozapine administration, implying that these genes play a significant role in clozapine-induced agranulocytosis. In addition, we found that clozapine is toxic to mesenchymal stromal cells in primary cultures. This indicates that impairment of bone marrow stromal cells during clozapine therapy may be a contributing factor to the drug´s toxicity.

8.2. Methodological limitations

A considerable limitation in the studies investigating the HLA-associations was the small numbers of patients in all patient groups. We only had eight patients with clozapine-induced agranulocytosis (neutrophil count $<0.5 \times 10^9$), although the possible HLA-A1 association in this patient group is of particular interest. Furthermore, the same HLA-associations may not be found in other populations with a different genetic background.

The study analyzing gene expression in blood leukocytes after clozapine administration included eight patients with schizophrenia who started clozapine treatment for the first time. This small number of patients is an obvious limitation. The patients used also other psychotropic drugs concomitantly, and their influence on the results cannot be excluded. It would therefore have been important to have a control group consisting of patients using antipsychotic drugs other than clozapine.

The study investigating the effect of clozapine on mesenchymal bone marrow stromal cells in primary cultures was also only a preliminary study

comprising a small number of experiments. Moreover, there was considerable variation in the luminescence emitted by both stromal cells and fibroblasts. Also the MSCs of individual bone marrow donors may differ in their sensitivity to clozapine. Finally, the cells only were incubated for only 24h. Considering that clozapine treatment typically lasts months or even years and that the onset of agranulocytosis is delayed, the effects in the cell cultures might have been more pronounced using a longer incubation time.

These limitations have been taken into account in the following section where the findings of each of the studies are discussed.

8.3 The impact of HLA haplotype on antipsychotic drug response in schizophrenia and the risk of clozapine-induced agranulocytosis

An association between responsiveness to antipsychotic drug treatment and HLA class I specificity was found. More than half of the patients refractory to first-generation antipsychotics but responsive to clozapine carried the HLA-A1 allele. This finding is consistent with some other studies which suggest that HLA-A1 may result in a poorer response to conventional antipsychotics (Smeraldi et al. 1976, Bersani et al. 1989, Alexander et al. 1990, Meged et al. 1999, Marchini et al. 2001, Lahdelma and Koskimies 2004). Moreover, a recent genome-wide association study implicated the major histocompatibility complex on chromosome 6p in schizophrenia (International Schizophrenia Consortium et al. 2009). Although the International Schizophrenia Consortium study was not able to assign the association to a specific HLA allele, haplotype or region, it is of interest that our study linked antipsychotic drug response in schizophrenia patients to the aforementioned region.

We reported that HLA-A1 positive patients not responding to first-generation antipsychotics have a good therapeutic outcome with clozapine and a relatively low risk of agranulocytosis. The study comprised of 26 patients with clozapine-induced granulocytopenia or agranulocytosis, 8 of whom had agranulocytosis. Notably, none of the patients with agranulocytosis carried the HLA-A1 allele. It is possible that clozapine-

induced agranulocytosis is particularly associated with the low frequency of this allele, as clozapine-induced granulocytopenia may represent a more heterogeneous group, where only some of the patients may actually develop agranulocytosis. As previously mentioned, clozapine-induced agranulocytosis and granulocytopenia may be distinct disorders with different etiological mechanisms (Flanagan and Dunk 2008). Of the 8 patients with agranulocytosis, 6 were females and 2 were men. The mean age in this group was 43.0 years, whereas in the group as a whole it was 34.9 years. These data are consistent with previous reports associating increasing age and female gender with an increased risk of clozapine-induced agranulocytosis.
We could also confirm an association between agranulocytosis and HLA-B16 that has been reported in earlier studies, one of which also included Finnish patients (Lieberman et al. 1990, Pfister et al. 1992, Claas et al. 1992). A slight increase in the frequency of HLA-B8 allele was seen in clozapine treated patients, but it did not extend to the HLA-DR3 allele. This is of interest as the HLA-A1, B8, DR3 haplotype is in high linkage disequilibrium and associates to a well-known susceptibility to autoimmune diseases. Finally, we found a new association with the HLA-A28 allele.

Theoretically, the association between HLA and responsiveness to drug therapy or the risk of developing clozapine-induced agranulocytosis may speak in favor of an immunomediated mechanism, but so far no such mechanism has been demonstrated convincingly (Opgen-Rhein and Dettling 2008). We suggest that HLA-A1 may be in linkage disequilibrium with susceptibility genes in the MHC region and serve as a marker for genes within the HLA area on chromosome 6. These genes, which are accountable for susceptibility, could be involved in schizophrenia, antipsychotic drug response and clozapine-induced agranulocytosis.

Linkage could be to other MHC-genes or non-MHC genes. It has been established that the MHC region has functions related to the immune system, but non-MHC genes may also influence immune response while encoding cytokine receptors or macrophage function (Warrens and Lechler 2000). An HLA allele could also be in linkage dysequilibrium to a susceptibility gene with functions other than immunological, such as with polymorphisms of NQO2, which may be involved in defective oxidative mechanisms implicated in clozapine-induced agranulocytosis (Ostrousky et al. 2003).

Moreover, selective linkage disequilibrium has been described between HLA-A and HLA-G, a non-classical class I antigen expressed by placental trophoblast cells (Ober et al. 1996, Debnath and Chaudhuri 2006). HLA-G may play an important role in the regulation and production of certain cytokines by immune cells in the uterus. Cytokine events at the maternal-fetal interface may be associated with prenatal immune challenge and the emergence of behavioral dysfunctions in adulthood (Meyer et al. 2008). The disturbance of HLA-G expression could, hypothetically, be connected to abnormalities in fetal brain development and contribute to the development of schizophrenia (Debnath and Chaudhuri 2006).
Taking the association between the HLA-A1 allele and antipsychotic drug response in schizophrenic patients we have described into consideration, the linkage disequilibrium between alleles at the HLA-A and-G locus is interesting. This could, hypothetically, select a subgroup of patients with schizophrenia, in whom immunological factors underpin the particular pattern of drug responsiveness and reduced susceptibility to clozapine-induced agranulocytosis. Cytokines are known to upregulate the expression of MHC class I proteins in neurons *in vitro* (Fujimaki et al. 1996). It has been postulated that changes in neuronal MHC class I expression during neurodevelopment might cause abnormalities in neuronal circuits leading to schizophrenia. Furthermore, adult cytokine-induced changes in MHC class I expression might acutely affect synaptic function during psychotic episodes (Debnath and Chaudhuri 2006). This hypothesis would provide an interesting mechanism by which HLA class I could select drug responsiveness during psychotic episodes in schizophrenia.

Our results may also suggest that HLA-A1 could be in linkage disequilibrium with genes on chromosome 6 predisposing to schizophrenia, defining a subgroup with a selective response to antipsychotics. HLA-A1 positive patients who are resistant to first-generation antipsychotics may have a relatively low risk of agranulocytosis and a good therapeutic outcome with clozapine.

8.4 Alterations in gene expression alterations after clozapine administration

We treated granulocyte precursor cells, HL-60 (derived from a human promyelocytic leukemia) with clozapine and compared their gene expression pattern with untreated HL-60 cells. After analyzing 588 genes known to be expressed in normal hematopoietic cells and hematological disorders, we found altered gene expression in 24 genes in the clozapine-treated cells compared with the untreated HL-60 cells. Four of these genes were selected for quantitative RT-PCR (real-time reverse transcriptase-polymerase chain reaction) analysis due to their implication in the maturation or apoptosis of granulocytes. The altered gene expressions in the HL-60 cells were validated and gene expression differences at 5 different time points of clozapine treatment were assessed in patient lymphocytes and granulocytes.

The kinetics were similar in both the patient leukocytes and the clozapine-treated HL-60 cells, which supports the hypothesis that clozapine plays a role in altering the gene expression pattern. The expression of MPO (myeloperoxidase precursor) was down-regulated, whereas that of MNDA (myeloid cell nuclear differentiation antigen) was up-regulated. These altered expression profiles continued in a linear curve through all time points, up to and including four months after start of treatment. Therefore it would seem likely that these two genes play a significant role in clozapine-induced agranulocytosis, especially as the risk of agranulocytosis is highest during the first 3 months of treatment. We also observed altered gene expression patterns of the FLT3LG (Fms-related tyrosine kinase 3 ligand) and ITGAL (antigen CD11A, lymphocyte function-associated antigen1) genes, which may suggest that they are also involved.

A number of studies have shown that clozapine is oxidized by activated neutrophilic granulocytes and bone marrow cells to a reactive nitrenium ion through the myeloperoxidase (MPO)- hydrogen peroxidase system (Ip et al. 2008). A study in schizophrenic patients with clozapine-induced agranulocytosis showed an increased frequency of homozygous carriers of low active polymorphism of MPO, indicating low levels of MPO-generated oxidants in those patients. The investigators suggested that the decrease of

MPO expression may cause changes in the activity balance of the NADPH-oxidase/MPO system, the major oxidation system in neutrophils. This could increase the amount of oxide radicals and toxic nitrenium metabolite count in neutrophils (Mosyagin et al. 2004). Our finding of decreased expression of MPO in granulocytes after clozapine administration is in line with this observation. A lower expression of the MPO gene may be caused by its direct inhibition of MPO transcription. Alternatively, clozapine treatment may select subclones of granulocytes with low natural MPO expression, while cells with high expression are eliminated by nitrenium ion toxicity.

Our observation suggests that the expression of the MPO and MNDA genes is altered in granulocytes after clozapine administration and this may have some bearing on clozapine-induced hematotoxic reactions in patients with schizophrenia. The altered gene expression patterns of the FLTT3LG and ITGAL genes may also suggest their involvement.

8.5 The effect of clozapine on primary cultures of human bone marrow stromal cells

We showed that, independent of bioactivation, clozapine is cytotoxic to primary cultures of mesenchymal human bone marrow stromal cells at a slightly supratherapeutic concentration of 10 µM. Strikingly, clozapine had a selective inhibitory effect on MSCs, whereas it had a growth-stimulating effect on primary human skin fibroblasts at the same concentration. Furthermore, we could not show any significant additional toxicity when using 10 µM of bioactivated clozapine. In contrast, bioactivation appeared to protect the cells from the toxic effects of clozapine. This observation differs from a study using immortalized human bone marrow stromal cell lines, where clozapine was cytotoxic only after bioactivation (Williams et al. 2000, Pereira and Dean 2006). Our findings suggest that direct cytotoxicity to bone marrow mesenchymal stromal cells may be one mechanism by which clozapine induces agranulocytosis.

The bioactivation of clozapine to a chemically reactive intermediate has been implicated as one of the main mechanisms by which clozapine induces agranulocytosis (Pereira and Dean 2006). A number of studies have shown that bioactivated clozapine at therapeutic concentrations of 1-3µM can

induce apoptosis and toxicity in neutrophils as well as in immortalized stromal cells lines. However, one study investigating the effect of clozapine adducts in rat bone marrow was unable to demonstrate myelotoxicity (Gardner et al. 2005). Clozapine-induced agranulocytosis may be a heterogeneous phenomenon with many etiological mechanisms (Uetrecht 1996, Gardner et al. 2005). It has been speculated that milder cases of white blood cell dyscrasia may represent increased sensitivity to the reactive metabolite (Uetrecht 1996). Theoretically, this could be the mechanism for some cases of granulocytopenia. On the other hand, the more serious conditions and fatal cases often occur within the first 3 months of treatment (Alvir et al. 1993, Atkin et al. 1996). It is feasible that this is an indication of a direct cytotoxic effect on bone marrow stromal cells.

Treatment with clozapine typically lasts months or even years. We therefore used a slightly supratherapeutic concentration of 10 μM of clozapine in our short-term (24h) toxicity assay. We hypothesize that the modest growth-inhibiting effects detected may be amplified in the bone marrow of patients undergoing long-term therapy with clozapine. Dose and serum levels of clozapine may not be directly associated with clozapine-induced agranulocytosis, although some reports suggest a dose-related aspect of this phenomenon (Flanagan and Dunk 2008a, Pessina et al. 2006). One study reported increased clozapine concentrations in the leukocytes of patients who developed clozapine-induced leukocytopenia, which was linked to changes or abnormalities in the cellular uptake of clozapine (Bergemann et al. 2007). It is possible that the MSCs take up clozapine more efficiently than human skin fibroblasts do. Nevertheless we were unable to measure the clozapine concentration in the cells. An alternative explanation could be that MSCs and fibroblasts metabolize clozapine along different pathways and therefore accumulate toxic compounds differently.

A striking finding in our study was the cytotoxic effect of clozapine on primary bone marrow stromal cells, in comparison to its growth-stimulating effect on primary human skin fibroblasts at the same concentration. This indicates that a direct cytotoxic effect on bone MSCs is one possible mechanism by which clozapine induces agranulocytosis.

9. CONCLUSIONS AND IMPLICATIONS

Drug response may subdivide patients with schizophrenia. We found an association between refractoriness to first-generation antipsychotics and response to clozapine and human leukocyte antigen A1. In addition, we showed that HLA-A1 may not only predict a good therapeutic response but select patients with a relatively low risk in developing clozapine-induced agranulocytosis. The biological explanations for these findings are still hypothetical, but they could imply a genetic association. HLA-A1 could be in linkage disequilibrium with a number of other genes involved in antipsychotic drug response and clozapine-induced agranulocytosis in patients with schizophrenia.

We found that treatment with clozapine induced altered gene expression of four genes implicated in the maturation or apoptosis of granulocytes: MPO (myeloperoxidase precursor), MNDA (myeloid cell nuclear differentiation antigen), FLT3LG (Fms-related tyrosine kinase 3 ligand) and ITGAL (antigen CD11A, lymphocyte function-associated antigen 1). These genes could either be directly involved in clozapine-induced agranulocytosis or could also be linked to other genes contributing to the condition.

Our results show that clozapine is cytotoxic to primary cultures of human bone marrow mesenchymal stromal cells at a slightly supratherapeutic concentration. This suggests that direct cytotoxicity to bone marrow stromal cells is one possible mechanism by which clozapine induces agranulocytosis. This may also be a general mechanism that contributes to drug-induced agranulocytosis, one of the most severe forms of idiosyncratic toxicity.

The results provide an encouraging basis on which to conduct further studies with larger numbers of Finnish-born patients and patients with other origins than the genetically homogeneous Finnish population, in order to confirm the HLA-association described. In addition, HLA-A1 could be a useful marker for further identification of the predisposing genes on chromosome 6. To confirm our findings on altered gene expression further evidence from a larger number of patients is required, together with more extensive gene screenings. Finally, studies are now needed that specifically

address the mechanisms of the toxic effect of clozapine on bone marrow stromal cells.

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Liisa Lahdelma

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